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**ISPITIVANJE STABILNOSTI SERIN- I
CISTEIN-PROTEAZA NA NISKIM
TEMPERATURAMA**

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**COLD STABILITY OF SERINE AND
CYSTEINE PROTEASES**

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LISTA SKRAĆENICA

AA - monomerni rastvor akrilamida i bisakrilamida
AB - amonijum-bikarbonat
AB-G - amonijum-bikarbonat sa glicerolom
AB-K - amonijum-bikarbonat sa lizinom
APS - amonijum-persulfat
ATR - umanjena ukupna refleksija (*engl.* Attenuated Total Reflectance)
BAPNA - N α -benzoyl-DL-arginin 4-nitroanilid-hidrohlorid
BSA - goveđi serum albumin (*engl.* Bovine Serum Albumin)
CBB - (*engl.* Coomassie Brilliant Blue)
CD - cirkularni dihiroizam
CDU - jedinica digestije kolagena (*engl.* Collagen Digestion Unit)
CTL9 - C-terminalni domen ribozomalnog proteina L9
CV - zapremina kolone (*engl.* Column Volume)
DEAE - dietilaminoetil-
DMSO - dimetilsulfoksid
DNA - dezoksiribonukleinska kiselina (*engl.* Deoxyribonucleic Acid)
E-64 - N-[N-(L-3-transkarboksiiran-2-karbonil)-L-Leucil]-agmatin
EDTA - etilendiamin tetraacetat
ESI - elektrosprej jonizacija
FT-IR - infracrvena spektroskopija sa Furijeovom transformacijom
GDU - jedinica digestije želatina (*engl.* Gelatin Digestion Unit)
HCl - hlorovodonična kiselina
HPLC - tečna hromatografija visokih performansi (*engl.* High Performance Liquid Chromatography)
IAA - jodoacetamid
IEF - izoelektrično fokusiranje
K - lizin
LC-MS - tečna hromatografija sa masenom spektrometrijom (*engl.* Liquid Chromatography - Mass Spectrometry)
MMTS - S-metil metantiosulfonat
NMR - nuklearna magnetna rezonanca (*engl.* Nuclear Magnetic Resonance)
PDB - proteinska banka podataka (*engl.* Protein Data Bank)
PEG - polietilen-glikol
PG - propilen-glikol
PMSF - fenilmetilsulfonil fluorid (*engl.* Phenylmethylsulphonyl Fluoride)
PUZ - pufer za pripremu uzorka
R - arginin
RP-HPLC - reverzno-fazna tečna hromatografija visokih performansi (*engl.* Reverse-phase High Performance Liquid Chromatography)

SAXS - rasejanje x-zraka pod malim uglom (*engl.* Small-angle X-ray Scattering)
SDS - natrijum-dodecil sulfat (*engl.* Sodium Dodecyl Sulfate)
SDS-PAGE - natrijum-dodecil sulfat poliakrilamidna gel elektroforeza (*engl.* Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)
STI - inhibitor tripsina iz soje (*engl.* Soybean Trypsin Inhibitor)
TCA - trihlorsirćetna kiselina (*engl.* Trichloroacetic Acid)
TEMED - tetrametiletildiamin
TLCK - tozil lizil hlorometan hidrohlorid
 T_m - temperatura topljenja (vrednost temperature na kojoj je 50% proteina u nativnoj konformaciji) (*engl.* Melting Temperature)
 T_c - temperatura na kojoj je 50% proteina u nativnoj konformaciji (*engl.* Temperature of Cold Denaturation)
TMF - peptidno mapiranje metodom otiska prsta nakon digestije tripsinom (*engl.* Trypsin Mass Fingerprinting)
TOF - vreme preleta (*engl.* Time-of-flight)
TPCK - tozil fenilalanil hlorometil keton
Tris - tris(hidroksimetil)aminometan

Ispitivanje stabilnosti serin- i cistein-proteaza na niskim temperaturama

SAŽETAK

Denaturacija globularnih proteina na niskim temperaturama predstavlja univerzalni fenomen. Narušavanje nativne strukture proteina usled izlaganja niskim temperaturama dešava se primarno kao posledica kolapsa hidrofobnog efekta, entropijskog faktora koji u najvećoj meri doprinosi stabilizaciji nativne strukture. Sekundarno, denaturacija proteina na niskim temperaturama posledica je slabljenja hidrofobnih interakcija u unutrašnjosti nativnog proteina. Ispitivanje stabilnosti proteina na niskim temperaturama ima, pre svega, fundamentalni značaj koji se ogleda u činjenici da bi detaljno razumevanje mehanizma denaturacije proteina na niskim temperaturama moglo značajno da doprinese razjašnjavanju jednog od najvažnijih problema savremene biohemije, problema uvijanja proteina.

Ispitivanje stabilnosti proteina na niskim temperaturama započeto je pre oko dve decenije. Međutim, direktnu denaturaciju proteina, koja je posledica niske temperature *per se*, teško je proučavati rutinski primenjivanim metodama, jer većina proteina ima tačke denaturacije ispod temperature mržnjenja vode. Razvojem savremenih FT-IR instrumenata, kao i primenom istih u određivanju promena sekundarnih struktura proteina, došlo je do povećanog interesovanja za proučavanjem denaturacije proteina izazvane niskim temperaturama.

Praktični značaj proučavanja ove problematike ogleda se u tome što razumevanje mehanizama denaturacije može pomoći u pronalaženju optimalnih uslova za skladištenje proteina pri kojima će se produžiti njihov vek trajanja. Poznavanje stabilnosti enzima naročito je važno u slučaju enzima koji se koriste u biotehnologiji, medicini ili nauci, kao što je slučaj sa sve četiri model proteaze koje su predmet istraživanja u okviru ove doktorske disertacije.

Najvažniji cilj koji je postavljen u ovoj studiji jeste pronalaženje objašnjenja za gubitak aktivnosti izazvan niskom temperaturom ispitivanih serin- i cistein-proteaza na nivou detaljnih strukturnih promena proteina, odnosno, pokazati da su, zapravo,

denaturacija proteaza i strukturni rearanžmani koji prate denaturaciju odgovorni za gubitak aktivnosti u mnogo većoj meri nego autoproteoliza. Dodatni cilj bilo je optimizovanje uslova za skladištenje komercijalno važnih proteaza na niskim temperaturama.

Praćenje stabilnosti proteina na niskim temperaturama nailazi na metodološka ograničenja kada je reč o primeni uobičajeno korišćenih metoda za ispitivanje stabilnosti proteina, kao što su spektroskopske metode i diferencijalna skenirajuća kalorimetrija. Stoga je u ovoj studiji pribegnuto pristupu izlaganja proteaza uzastopnim ciklusima zamrzavanja/odmrzavanja u cilju praćenja uticaja temperatura ispod nule na strukturu proteaza. Rezultati praćenja promena primarne strukture model proteaza koji su dobijeni kao deo ove disertacije, ukazuju da autoproteoliza ne može biti odgovorna za visoke procenite gubitka aktivnosti, (čak do 75% u slučaju papaina, preko 40% u slučaju ficina i oko 60% u slučaju tripsina u kiselim uslovima) nakon šest ciklusa zamrzavanja/odmrzavanja. Praćenje strukturnih perturbacija na nivou sekundarne i tercijarne strukture pokazalo je da nakon šest do sedam uzastopnih ciklusa zamrzavanja/odmrzavanja proteaze gube elemente native sekundarne strukture (α -heliks i neuređene strukture) u korist β -pločica (intramolekulskih u slučaju tripsina u kiselim uslovima). Naročito je izražen porast sadržaja intermolekulskih β -pločica (u slučaju papaina i ficina) koje predstavljaju strukturne elemente neophodne za agregiranje. Agregiranje je potvrđeno gel-filtracijom. Opisani trendovi promena sekundarnih struktura detektovani su u literaturi i za druge proteine denaturisane niskim temperaturama, odnosno niskom pH vrednošću, indicirajući da je inaktiviranje papaina, ficina i tripsina (u kiselim uslovima) na niskoj temperaturi posledica denaturacije.

U slučaju tripsina za sekvenciranje, proteaze od najvećeg komercijalnog značaja u ovoj studiji, predložen je alternativni način skladištenja na niskoj temperaturi u rastvornom obliku kojim se izbegava denaturacija i ograničava autoproteoliza. Blago alkalna pH vrednost (u blizini optimalne vrednosti za aktivnost tripsina) uz dodatak krioprotektivnih agenasa (glicerola i lizina) za koje je poznato da stabilizuju proteine (mehanizmom preferencijalne potisnutosti sa površine proteina favorizujući nativnu konformaciju), dovela je do efikasnog očuvanja native strukture tripsina. Takođe, inhibicijom autoproteolitičke aktivnosti u prisustvu lizina koji okupira vezujuće mesto

tripsina, ograničena je i autoproteoliza. Tripsin skladišten na niskoj temperaturi na pH vrednosti od 8,0 uz dodatak glicerola ili lizina, pokazao je efikasnost identičnu netretiranom nativnom tripsinu u metodi identifikovanja BSA peptidnim mapiranjem, što sugeriše da skladištenje tripsina u blago alkalnim uslovima uz dodatak krioprotektanata može da produži njegov vek trajanja.

Ključne reči: proteaze, stabilnost na niskim temperaturama, denaturacija, agregiranje, enzimska aktivnost, autoproteoliza, FT-IR spektroskopija, tripsin za sekvenciranje

Naučna oblast: Biohemija

Uža naučna oblast: Strukturna biohemija

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Cold stability of serine and cysteine proteases

ABSTRACT

Cold denaturation of globular proteins represents a universal phenomenon. Disruption of native structure at low temperatures primarily happens as a consequence of the collapse of the hydrophobic effect, an entropy parameter which represents a main driving force for protein folding. Secondly, protein denaturation at low temperatures is a consequence of the weakening of hydrophobic interactions in the interior of a protein's three dimensional structure. Investigation of the cold denaturation of proteins has fundamental importance, because detailed understanding of the mechanism of cold denaturation could contribute to a great extent toward elucidating one of the most challenging problems of contemporary structural biochemistry: the protein folding problem. Investigation of the cold stability of proteins began two decades ago. Direct cold denaturation, which is a consequence of low temperature *per se*, is difficult to investigate using routine methods since the majority of proteins have cold denaturation points well below 0 °C. However, the development of modern FT-IR instruments and their application in secondary structure determination has led to increased interest in the investigation of the cold stability of proteins.

The practical importance of this topic reflects the fact that understanding structural rearrangements induced by cold denaturation could help define optimal conditions for protein storage in order to prolong their shelf lives. The cold stability of proteins is especially important for enzymes used in biotechnology, medicine or research: which is the case for all four model proteases investigated in this dissertation.

The overall goal of this study was to explain the dramatic loss of serine and cysteine proteases activity induced by low temperature, by showing that cold denaturation and subsequent massive structural rearrangements are responsible for this loss, rather than autolysis. In addition, optimal cold storage conditions were defined for commercially important proteases.

In this work, repeated freeze-thaw cycles were used to analyse the influence of sub-zero temperatures on protease structures, due to methodological limitations in monitoring protein stability at sub-zero temperatures by commonly used techniques

such as spectroscopy and differential scanning calorimetry. Results from monitoring the primary structure stability of model proteases obtained as a part of this dissertation suggest that autoproteolysis cannot be the cause for the dramatic activity losses (as large as 75% in the case of papain, above 40% in the case of ficin and around 60% in the case of trypsin in acidic conditions) observed after six freeze-thaw cycles. Secondary/tertiary structure perturbations after six-to-seven freeze-thaw cycles suggest that these model proteases lose part of their native secondary structure elements (mainly α -helices and random coils) in favor of a β -sheet conformation (intramolecular in the case of trypsin in acidic conditions). An especially large increase was detected for intermolecular β -sheets (in the case of papain and ficin) which represents a structural element necessary for aggregation. Aggregation of cold denatured cysteine proteases was shown by size-exclusion chromatography. Similar trends in secondary structure changes were detected in the literature for other proteins denatured by low temperature and/or low pH values, indicating that inactivation of papain, ficin and trypsin (in acidic conditions) was a consequence of cold denaturation.

Sequencing grade trypsin, being the most important commercial protease of this study, was chosen for optimization of cold storage conditions. An alternative protocol for cold storage of trypsin in solution is proposed which circumvents cold denaturation and limits autoproteolysis. Slightly alkaline conditions (close to the optimum pH value of trypsin, pH around 8) with the addition of cryoprotectants (glycerol or lysine which are known to stabilise proteins in solution by preferential exclusion from the protein surface favoring the native state) led to complete preservation of native structure. In the case of lysine as a cryoprotectant, autoproteolysis was inhibited as well. After seven cycles of cold storage at pH around 8 with the addition of cryoprotectants, trypsin was as efficient as untreated trypsin in trypsin mass fingerprinting of BSA, suggesting that proposed conditions could prolong its shelf life.

Key words: protease, cold stability, denaturation, aggregation, enzyme activity, autoproteolysis, FT-IR spectroscopy, sequencing grade trypsin

Scientific field: Biochemistry

Scientific subfield: Structural biochemistry

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1. UVOD

Ispitivanje stabilnosti proteina na niskim temperaturama ima višestruki, i fundamentalni i praktični značaj. U cilju razumevanja jednog od najvažnijih nerešenih problema strukturne biohemije, načina uvijanja proteina, veoma je važno razumeti stabilnost/denaturaciju proteina na niskim temperaturama. Denaturacija proteina na niskim temperaturama predstavlja fenomen koji se tek u poslednje dve decenije intenzivno proučava, a koji ima važne implikacije na poremećaje i oboljenja izazvane upravo pogrešnim uvijanjem proteina i formiranjem amiloidnih agregata [1, 2]. Što se praktičnog aspekta tiče, enzimi od komercijalne važnosti često se skladište na niskim temperaturama. Upravo iz tog razloga, kao i zbog upotrebe na niskim temperaturama, stabilnost enzima jedan je od ključnih faktora koji ograničava njihovu upotrebu u komercijalne svrhe [3]. Razumevanje stabilnosti proteina na niskim temperaturama od suštinske je važnosti i za razumevanje adaptacije živih bića na ekstremno niske temperature [4].

Poslednjih decenija pretpostavlja se da proteaze tokom skladištenja na niskim temperaturama gube aktivnost usled autoproteolize. Stoga proizvođači proteolitičkih enzima preporučuju njihovo skladištenje u uslovima udaljenim od pH optimuma, bez obzira na to što su takvi enzimski preparati uglavnom ograničeni na mali broj ciklusa zamrzavanja/odmrzavanja. Kako se mehanizam i razumevanje fenomena denaturacije proteina na niskim temperaturama intenzivno ispituju tek poslednjih dvadesetak godina, nameće se interesantna hipoteza: da izlaganje proteaza niskim temperaturama (ispod nule) u uslovima koji su daleko od optimalnih, dovodi do povećane inaktivacije usled denaturacije (u većoj meri nego gubitak aktivnosti usled autoproteolize). Ispitivanje navedene hipoteze predstavljalo je jedan od glavnih ciljeva ovog istraživanja i sprovedeno je na cistein- i serin-proteazama koje su predstavljale model sisteme ove studije.

Pionirska istraživanja ustanovila su osnovne principe zbog kojih dolazi do denaturacije proteina na niskim temperaturama [1]. Veliki broj studija o problematici denaturacije izazvane niskim temperaturama može se pronaći u naučnoj literaturi [5-9]. Međutim,

iako denaturacija proteina na niskim temperaturama predstavlja univerzalni fenomen za globularne proteine, može se primetiti da ne postoje univerzalni putevi kojima se dešava denaturacija na niskim temperaturama. Nepostojanje optimalnog metodološkog pristupa, kao i do sada otkriveno heterogeno ponašanje različitih proteina na niskim temperaturama, govori u prilog kompleksnosti ove problematike, ali i potrebi za dodatnim istraživanjima u okviru iste.

Glavni cilj ovog istraživanja predstavlja pronalaženje strukturnog objašnjenja za inaktiviranje enzima niskim temperaturama, kako bi se, s jedne strane, pružio doprinos fundamentalnim istraživanjima problematike stabilnosti i denaturacije proteina na niskim temperaturama, a, sa druge strane, ponudilo rešenje za povećavanje stabilnosti komercijalnih enzima. Otkrivanje uzroka destabilizacije proteina niskim temperaturama može imati direktne implikacije na rešavanje problema inaktiviranja enzima i pronalaženje načina za adekvatno čuvanje i produženje roka trajanja komercijalnih enzima.

U okviru ove disertacije po prvi put je pokazano da proteaze, nasuprot opštem verovanju koje je rasprostranjeno u naučnoj literaturi, bivaju inaktivirane tokom skladištenja na niskoj temperaturi prevashodno usled denaturacije izazvane niskom temperaturom i pratećim stresovima povezanim sa formiranjem leda u vodenom rastvoru, a ne autoproteolizom. Osim toga, pokazano je da sva četiri ispitana enzima, koja su predmet ove studije, pokazuju sličan trend strukturnih promena koje su uzrok inaktiviranja. Naime, strukturni rearanžmani podrazumevaju povećavanje sadržaja β -pločica na račun drugih nativnih sekundarnih struktura koje bivaju narušene (α -heliks i neuređene strukture). Međutim, pronađene su i razlike u obrascima denaturacije cistein- i serin- proteaza, koje bi mogle da doprinesu objašnjenju fenomena denaturacije na niskim temperaturama u zavisnosti od obrazaca uvijanja različitih proteina.

2. TEORIJSKI DEO

2.1. Struktura proteina i konformacioni prelazi

Proteini su kompleksni biomakromolekuli sastavljeni od aminokiselina koje su kovalentno povezane peptidnim vezama. Značaj proteina ogleda se u veoma raznovrsnim funkcijama koje proteini obavljaju u svakom biološkom procesu. Obavljanje važnih i kompleksnih funkcija u živim sistemima proteini duguju svojoj tačno definisanoj trodimenzionalnoj strukturi (nativnoj strukturi) u koju se spontano uvijaju [10].

2.1.1. Nivoi strukture proteina

Struktura proteina hijerarhijski je podeljena na različite nivoe kompleksnosti. Tako primarna struktura proteina podrazumeva kovalentne veze u proteinu, preciznije, aminokiselinsku sekvencu polipeptidne kičme, kao i položaj disulfidnih mostova između ostataka cisteina. Sekundarne strukture proteina odnose se na lokalno uvijanje (relativno) bliskih delova polipeptidnog niza u formu α -heliksa, β -pločice ili zavijutaka, dok finalna prostorna konformacija čitavog polipeptidnog niza predstavlja tercijarnu strukturu, kao najviši nivo strukture proteina koji su sastavljeni od jednog polipeptidnog lanca. Ukoliko je protein izgrađen od dva ili više polipeptidnih lanaca koji se nezavisno sintetišu, najviši oblik strukture datog proteina označava se kao kvaternarna struktura [11].

2.1.2. Uvijanje proteina

Svaki protein ima jedinstvenu trodimenzionalnu strukturu koja omogućava obavljanje specifične funkcije u organizmu. Od trenutka otkrića strukture proteina javilo se interesovanje naučnika za razjašnjavanje mehanizma uvijanja polipeptidnih lanaca u tačno određenu konformaciju koja je kao takva sposobna da obavlja određenu aktivnost/funkciju. Uvijanje tako ogromnih molekula iz linearnog niza aminokiselina u jedinstvenu, tačno određenu trodimenzionalnu strukturu (nativnu strukturu), jedan je od najvećih, još uvek nerešenih problema strukturne biohemije. Naučni časopis *Science* je 2005. godine objavio da je problem mehanizma uvijanja proteina svrstan u jedan od 125 najvećih nerešenih problema u nauci [2].

Proučavanje mehanizma uvijanja proteina (kao i suprotnog procesa, denaturacije proteina) i razjašnjavanje stabilnosti strukture proteina ne samo da predstavlja fundamentalno rešavanje jedne od najvećih misterija nauke, već bi rešavanje ove naučne problematike imalo direktne implikacije na mnoge konkretne i važne probleme. Naime, strukturne distorzije proteina u osnovi su mnogih bolesti, kao što je Alchajmerovo oboljenje, Parkinsonova bolest, spongiformne encefalopatije i mnoge druge za koje još uvek nije pronađen efikasan lek [12-14]. Pored toga, razumevanje načina na koji se proteini uvijaju omogućilo bi predviđanje trodimenzionalne strukture proteina na osnovu sekvence aminokiselina, kao i *de novo* sintezu, odnosno dizajniranje proteina sa novim funkcijama [15].

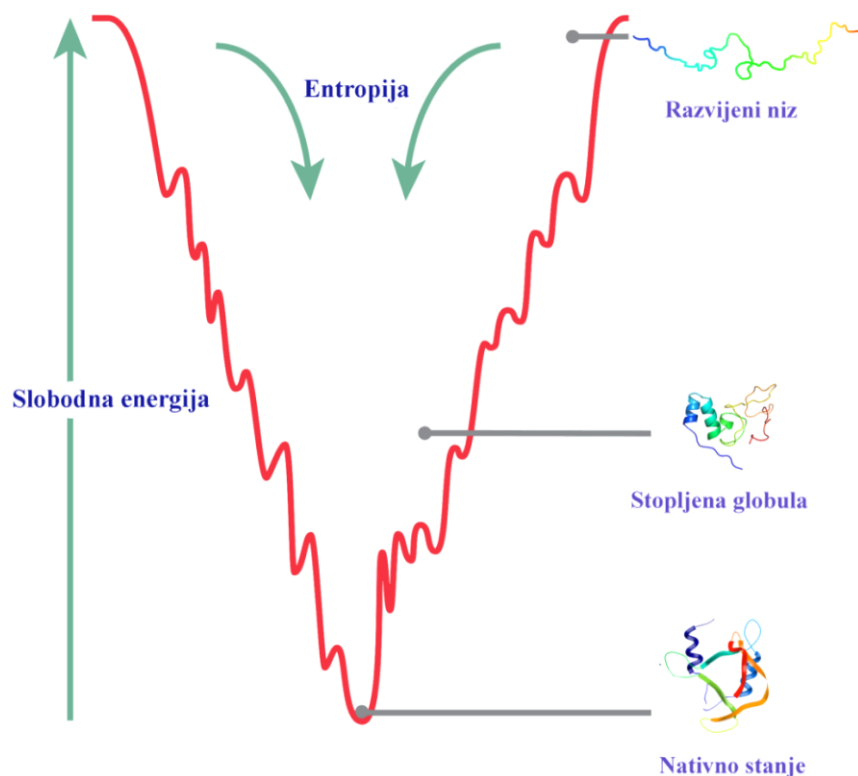
2.1.3. Kinetika uvijanja proteina

Mogućnost kristalizacije sve većeg broja proteina pokazala je da je njihova konformacija jedinstvena i tačno određena, jer samo kao takva može dati diskretne jedinice kristala tačno određene geometrije. Anfinsen je jedan od naučnika koji je dao veliki doprinos na putu ka razjašnjavanju procesa uvijanja proteina postulirajući takozvanu termodinamičku teoriju uvijanja proteina. On je eksperimentalno proučavao denaturaciju/renaturaciju ribonukleaze i na osnovu datog model sistema izveo tri ključna zaključka: informacija o trodimenzionalnoj strukturi proteina zapisana je u aminokiselinskoj sekvenci; nativna konformacija proteina je termodinamički stabilnija od razvijene; nativna konformacija je termodinamički najstabilnija konformacija [16, 17]. Ali, na koji način se informacija sadržana u redosledu aminokiselina prevodi u njihov prostorni raspored, pitanje je koje se u nauci kolokvijalno označava kao „druga polovina genetskog koda“. Za dešifrovanje ovog koda potrebno je odgovoriti na mnoga pitanja. Za početak, da li je proces uvijanja proteina vođen termodinamički ili kinetički? Koliko traje proces uvijanja? Kako se inicira ovaj proces? Da li je nativna struktura zaista najstabilnija [18]?

Proteini su izgrađeni od velikog broja aminokiselinskih ostataka sa stotinama kovalentnih veza. Oko velikog broja kovalentnih veza u aminokiselinskim ostacima proteina moguća je slobodna rotacija, te bi sledilo da, hipotetički, protein može da zauzme ogroman broj konformacija. U razvijenoj konformaciji svaki aminokiselinski ostatak zauzima bilo koju od 3 dozvoljene kombinacije ϕ i ψ uglova (dozvoljene oblasti

Ramačandranovog dijagrama). Polipeptidni niz od 100 aminokiselinskih ostataka bi mogao da zauzme 3^{100} ili 10^{48} konformacija. Međutim, nativni molekul proteina nalazi se u jednoj jedinstvenoj konformaciji (jedna određena kombinacija ϕ i ψ uglova) [19]. Levintal je prvi izračunao da kada bi takav protein nasumično isprobavao moguće konformacije na putu do nativne konformacije, proces njegovog uvijanja u nativnu strukturu trajao bi 10^{50} godina, dok u *in vitro* uslovima traje nekoliko milisekundi [20]. Ovaj postulat, koji kasnije postaje poznat pod nazivom „Levintalov paradoks“, otvorio je nova pitanja. Naime, i sam Levintal je predložio da brzo uvijanje proteina, može biti objašnjeno time da prvi koraci uvijanja podrazumevaju simultano formiranje većeg broja uvijenih jezgara. On je pretpostavio da najpre dolazi do formiranja manjih uvijenih segmenata uspostavljanjem interakcija između bliskih regiona polipeptidnog lanca, koji dalje usmeravaju uvijanje čitavog proteina [20]. Na Levintalov paradoks kasnije se nadovezuje i koncept takozvanog „levka uvijanja proteina“ koji daje prikaz energetskih površina uvijanja, odnosno termodinamičko i kinetičko ponašanje proteina na putu od razvijenog niza do nativne strukture. Ilustrovani prikaz levka uvijanja prikazuje smanjenje sadržaja energije i entropije kako proces uvijanja proteina odmiče, ali je i kinetika opisana nagibom levka. Danas je prihvaćena teorija da je postojanje trodimenzionalne strukture proteina pod termodinamičkom kontrolom, ali da se ona dostiže prolaskom kroz intermedijarna stanja, što je kinetički kontrolisano [18].

Tokom dugog perioda istraživanja mehanizma uvijanja proteina, predloženo je nekoliko različitih modela uvijanja proteina. U modelu „difuzije i sudara“ (*engl.* diffusion collision; framework) predloženo je da u prvim fazama uvijanja dolazi do uspostavljanja sekundarnih struktura između bliskih ostataka aminokiselina, a da potom sledi formiranje viših nivoa strukture. Suprotno tome, predložen je model takozvane „kondenzacije oko hidrofobnog jezgra“ u kome inicijalni korak predstavlja formiranje kompaktnog jezgra proteina koje je vođeno hidrofobnim efektom [21]. Jedan od najnovijih modela koji su predložili Dil i saradnici podrazumeva kombinaciju ova dva modela: simultano formiranje elemenata sekundarnih struktura i hidrofobnog kolapsa (model nukleacije i kondenzacije). Ono što ostaje nerazjašnjeno je da li uopšte postoji jedinstvena putanja uvijanja proteina ili je verovatnije da postoji veći broj mogućih [18]. Najnovija teorija koja kombinuje i termodinamičke i kinetičke efekte u uvijanju i agregiranju proteina naziva se teorija energetskog reljefa (slika 2.1) [2].



Slika 2.1. Šematski prikaz teorije energetskeg reljefa, odnosno relativnih stabilnosti različitih konformacionih stanja proteina tokom procesa uvijanja proteina.

Navedena teorija prikazuje se šematski u vidu takozvanog „levka uvijanja“ i pokazuje da razvijeni niz proteina ima najviši sadržaj energije, drugim rečima, da je najnestabilnije stanje. Idući različito strmim kanalisanim putanjama ka smanjenju sadržaja slobodne energije, protein prolazi kroz intermedijerna stanja delimično uvijene strukture koja su predstavljena kao lokalni energetske minimumi. Lokalni minimumi su kinetički stabilni intermedijeri uvijanja jer su razdvojeni barijerama aktivacione energije od nativnog stanja koje je termodinamički stabilnije (nižeg sadržaja energije). Nativno stanje proteina odlikuje se i najmanjom entropijom i često se predstavlja kao jedna diskretna tačka na dnu šeme energetskeg reljefa, dok se razvijeni niz karakteriše najvišom entropijom i predstavlja se kao široka površina na vrhu šeme (slika 2.1) [22].

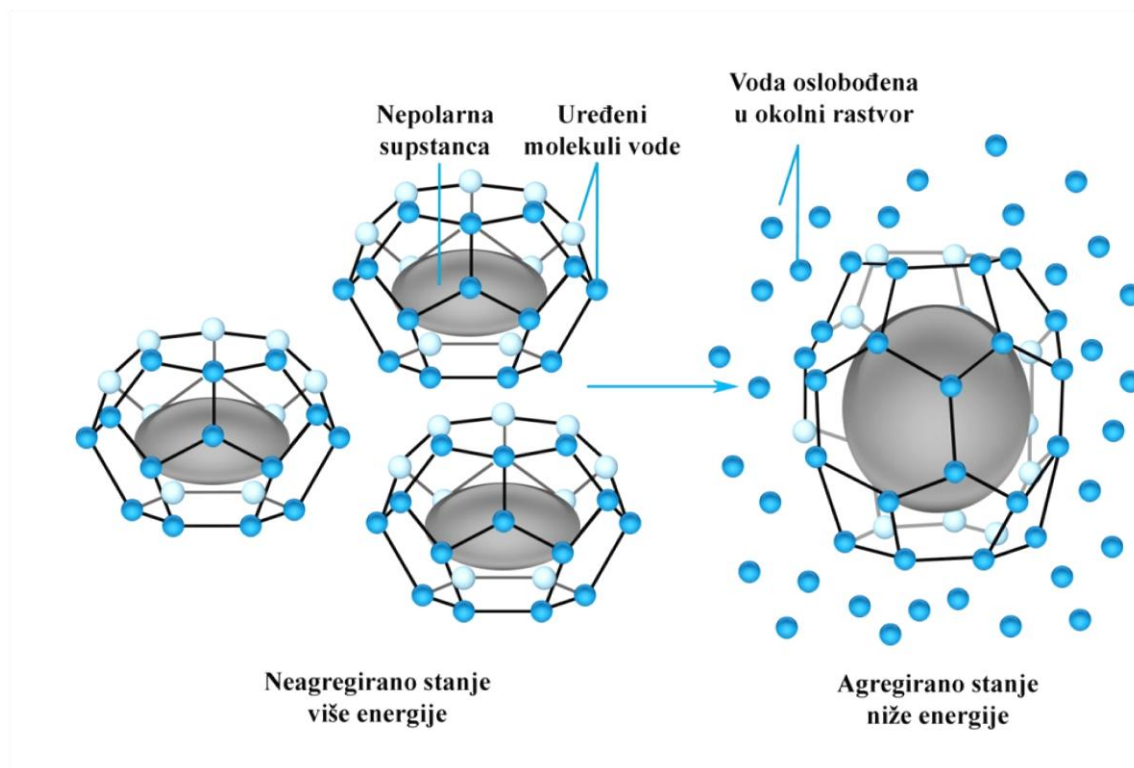
2.1.4. Termodinamika uvijanja proteina

Rezultati Anfinsenovog eksperimenta nedvosmisleno pokazuju da je nativna konformacija proteina stabilnija od razvijene konformacije [17]. Međutim, nativni proteini su samo marginalno stabilni. Razlika u sadržaju slobodne energije između nativnog i razvijenog proteina veoma je mala i kreće se u intervalu od 20 do 65 kJ/mol [23]. Ovako mala razlika u promeni slobodne energije između nativne i denaturisane konformacije proteina posledica je suprotstavljenih faktora koji stabilizuju jedno ili drugo stanje. Gibbs-Helmholcova jednačina:

$$\Delta G = \Delta H - T\Delta S$$

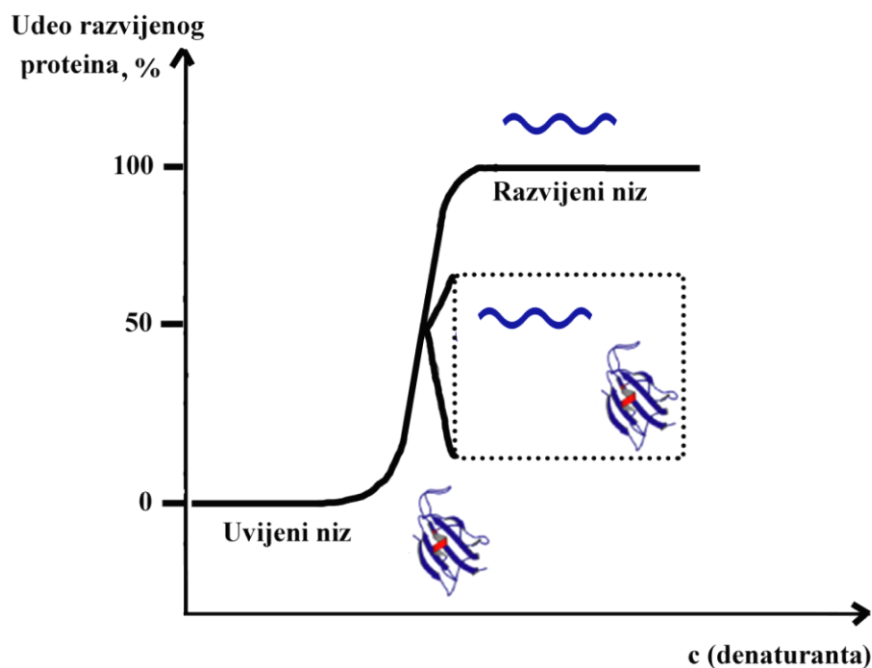
gde ΔG predstavlja razliku u Gibsovoj slobodnoj energiji, ΔH razliku u entalpiji, a ΔS razliku u entropiji između denaturisanog i nativnog proteina, daje nam odgovore na postavljena pitanja [24]. Entalpijski faktor (ΔH) odnosi se na uspostavljanje i raskidanje slabih nevezivnih interakcija između različitih delova polipeptida koji su došli u bliski kontakt tokom uvijanja proteina, kao i između polipeptida i vode. U slabe nevezivne interakcije spadaju hidrofobne interakcije, vodonične veze i elektrostatičke interakcije. Iako su nevezivne interakcije mnogo slabije od kovalentnih veza, prosečan protein gradi veliki broj ovakvih interakcija koje stabilizuju nativnu konformaciju proteina. Prilikom uvijanja proteina u nativnu strukturu dolazi do uspostavljanja novih intramolekulskih interakcija, ali isto tako i do raskidanja velikog broja interakcija koje su postojale između razvijenog niza i vode. Zato je sumarno entalpijski faktor često blizak nuli i ne doprinosi mnogo stabilizaciji nativne konformacije [25]. Konformaciona entropija odnosi se na razliku u stepenu neuređenosti razvijenog niza i nativne konformacije, tako da ovaj faktor ne ide u korist nativne konformacije, jer razvijeni niz ima mnogo veći broj stepeni slobode [26]. Međutim, slučaj nije takav kada se posmatra entropija vode. Molekuli vode formiraju visoko uređenu strukturu klatrata oko razvijenog niza okružujući izložene hidrofobne ostatke aminokiselina. Kada se protein uvije, dolazi do narušavanja uređene strukture klatrata usled grupisanja hidrofobnih ostataka aminokiselina u unutrašnjosti proteina, odnosno van kontakta sa vodom. Time dolazi do velikog porasta entropije vode, te ovaj efekat (koji se označava kao hidrofobni efekat) u najvećoj meri doprinosi stabilizaciji nativne strukture proteina [27, 28]. Hidrofobni

efekat prikazan je na slici 2.2. na primeru spajanja više lipidnih kapljica u lipidnu kap u vodenom rastvoru.



Slika 2.2. Šematski prikaz hidrofobnog efekta.

Kao model sistemi proučavanja mehanizma uvijanja/denaturacije proteina najčešće su korišćeni jednostavniji proteini, odnosno mali globularni proteini jednodomske strukture. Smatralo se da je uvijanje/razvijanje ovakvih proteina nužno reverzibilan, visoko kooperativan proces, što znači da nema stabilnih intermedijera, već samo prelaz između dva stanja (uvijenog i razvijenog) (slika 2.3) [29, 30].



Slika 2.3. Sigmoidna kriva opisuje kooperativni prelaz iz uvijenog u razvijeni niz proteina bez stabilnih intermedijera.

Međutim, kod većih proteina složenije strukture, na putu od potpuno razvijenog polipeptidnog niza do finalne nativne strukture postoje intermedijarna stanja delimične uređenosti [31]. Proces uvijanja, dakle, nije visoko kooperativan. Stabilni ravnotežni intermedijeri procesa uvijanja proteina eksperimentalno su identifikovani i nazivaju se stanjima stopljene globule [32].

Stopljena globula karakteriše se značajnim sadržajem sekundarnih struktura, ali joj nedostaje definisana i postojana tercijarna struktura za razliku od nativne forme. Stopljena globula je po kompaktnosti slična nativnoj konformaciji, ali za razliku od nje ima više izloženih hidrofobnih površina i tendenciju za agregiranjem [31, 33]. Time je zaključeno da je formiranje nativne funkcionalne konformacije u konkurenciji sa agregiranjem [34].

2.1.5. Denaturacija proteina i nenativno agregiranje proteina

Denaturacija proteina podrazumeva narušavanje nativne konformacije usled izlaganja proteina promenjenim uslovima okruženja. Struktura izolovanih proteina, koji bivaju otrgnuti od svog prirodnog miljea u kome nativno funkcionišu, lako se može narušiti

nizom stresnih uslova kao što su povišena ili niska temperatura, prisustvo organskih rastvarača, haotropnih agenasa, detergenata, naglim promenama pH vrednosti itd. Protein brzo gubi svoju nativnu konformaciju i, generalno posmatrano, menja način uvijanja i postaje neaktivan [14, 35]. Iako se smatralo da je denaturacija sinonim za potpuno razvijanje proteina, odnosno da je u pitanju prelaz iz nativne strukture u potpuno neuređeni niz (bez sekundarnih struktura), pokazano je da se, osim u slučaju denaturacije haotropima, zapravo dešava nastajanje (delimično) pogrešno uvijenih formi [30]. Denaturacija, dakle, najčešće znači promenu obrasca vodoničnih veza pri čemu nastaju delimično uvijena stanja u kojima preovladava samo jedna sekundarna struktura. Najčešće se u denaturisanom obliku javlja promena sekundarnih struktura iz α -heliksa u izdužene lance β -pločica [36].

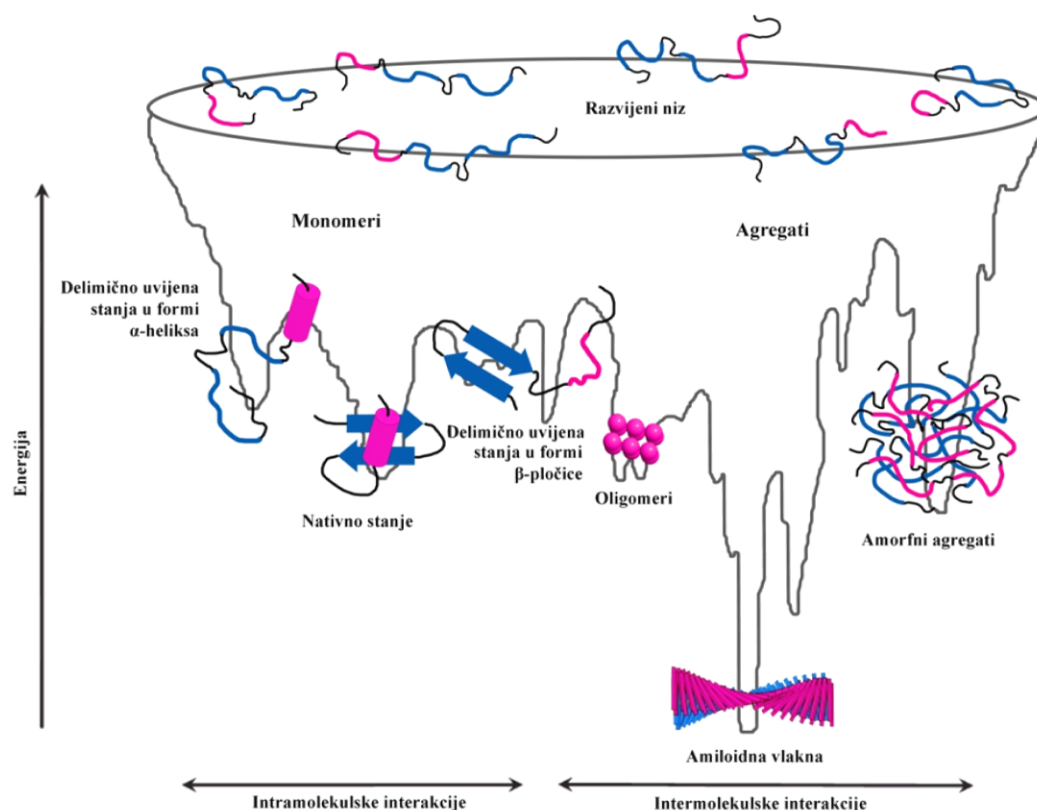
Delimično razvijene strukture (denaturisane strukture) su nestabilne i obično pokazuju tendenciju za agregiranjem usled izlaganja nepolarnih ostataka aminokiselina vodi i formiranja većeg broja β -pločica [14]. Proces denaturacije proteina biće idealno kooperativan prelaz između nativnog i razvijenog stanja jedino u prisustvu haotropa kao denaturanata (o čijem će mehanizmu denaturacije kasnije biti reči) ili ako je koncentracije proteina dovoljno niska da ne dođe do agregiranja [30].

Dakle, sumarno posmatrano, proces denaturacije proteina može podrazumevati širok spektar konformacija, od potpuno razvijenih/neuređenih do veoma struktuiranih konformacija koje se razlikuju od nativne, u zavisnosti od uslova pod kojima se dešava denaturacija [32]. Sveobuhvatna definicija denaturacije bi, stoga, glasila: tranzicija konformacije proteina iz uređene, odnosno, nativne u izmenjenu/nefunkcionalnu [36].

2.1.6. Proširena teorija energetskog reljefa

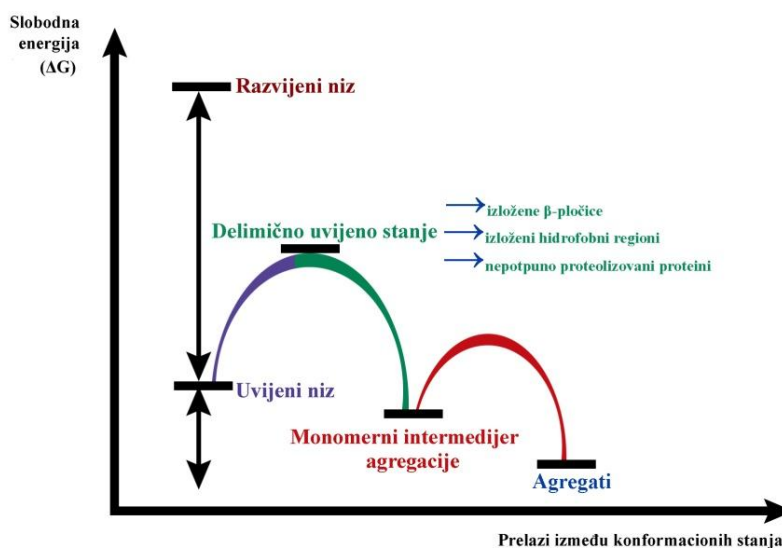
Kako opisuje teorija energetskog reljefa, uvijanje proteina dešava se prolaskom kroz uske putanje (uz smanjenje sadržaja slobodne energije) preko intermedijera uvijanja, sve do najstabilnijeg stanja (slika 2.1). Proširena varijanta teorije energetskog reljefa objašnjava i uvijanje i agregiranje proteina [34]. Prikazana šema (slika 2.4) pokazuje da, ukoliko se delimično uvijena stanja karakterišu povišenim sadržajem β -pločice, ona pod određenim uslovima okruženja (uz visoku barijeru aktivacione energije) mogu preći u različite forme agregata. Sve forme agregata karakterišu se nižim sadržajem slobodne

energije od native strukture. Manje kontrolisani uslovi okruženja dovode do formiranja neuređenih agregata amorfne strukture, dok ukoliko su uslovi više kontrolisani može doći do formiranja najstabilnije strukture proteina, amiloidnih vlakana [22].



Slika 2.4. Šematski prikaz relativnih stabilnosti različitih konformacionih stanja proteina tokom procesa uvijanja/denaturacije. Preuzeto i prilagođeno iz [22].

Proces agregiranja proteina podrazumeva najpre reverzibilno formiranje jezgra nukleacije sa kojim dalje interaguju denaturisani proteini, sve dok se ne pređe kritična veličina, nakon čega agregiranje postaje ireverzibilno uz stvaranje krupnih agregata koji se izdvajaju iz rastvora kao precipitat (slika 2.5). Smatra se da je agregiranje inicirano delovima proteina sa hidrofobnim aminokiselinskim ostacima, izloženim β -pločicama i niskim neto naelektrisanjem. Inicijalne forme proteina od kojih kreće proces agregiranja mogu biti pogrešno uvijene forme proteina, kao i razvijeni polipeptidni nizovi [37].



Slika 2.5. Šematski prikaz energetske razlike između razvijenog niza, nativnog stanja i agregata.

Nastali agregati se mogu razlikovati po svojoj strukturi. Oni mogu biti pravilni oligomerni agregati, neuređeni amorfni agregati, ili visoko struktuirani amiloidni fibrili [38].

2.2. Kozmotropi i haotropi

Prisustvo drugih molekula (aditiva) u vodenom rastvoru proteina bitan je faktor koji utiče na stabilnost native strukture proteina. Prema svom efektu na stabilnost proteina, aditivi se mogu podeliti na kozmotrope i haotrope. Kozmotropski efekat označava stabilizujući efekat na nativnu strukturu proteina, dok je haotropni efekat suprotnost – vodi ka destabilizaciji native strukture proteina. Suštinska razlika je u tome što kozmotropi bivaju preferencijalno potisnuti sa površine proteina, ne interagujući sa hidratacionim ljuskama proteina i favorizujući njihovu uvijenu konformaciju. Haotropi, s druge strane, preferencijalno interaguju sa površinom proteina i/ili hidratacionom ljuskom proteina, pri čemu favorizuju razvijeno stanje proteina [39].

2.3. Termalna denaturacija proteina

Koncept denaturacije proteina na povišenim temperaturama, budući da je intuitivno prihvatljiv, nastao je mnogo ranije od koncepta denaturacije na niskim temperaturama. Naime, u svakom procesu koji je indukovao povišenjem temperature, doći će do apsorpcije toplote i povećanja neuređenosti sistema. Tako će i struktura proteina biti

narušena usled zagrevanja. Međutim, nije se tako smatralo i za niske temperature. Kao logično nametalo se shvatanje da će sniženjem temperature protein zadržati svoju uređenu strukturu, jer će se uticaj nepovoljnog faktora entropije polipeptida smanjivati. Vremenom su eksperimenti pokazali potpuno suprotnu sliku: denaturacija proteina na niskim temperaturama opšti je fenomen. Ovo je i danas veoma zanimljivo pitanje u proteinskoj biohemiji bez čijeg rešavanja neće biti moguće potpuno razumevanje uvijanja proteina i njihove stabilnosti [1].

2.3.1. Denaturacija proteina na niskim temperaturama

Denaturacija proteina na niskim temperaturama pominje se od 1990. godine u radu Privalova [1]. Denaturacija proteina na niskim temperaturama dešava se kao posledica niske temperature *per se*, ali i kao posledica stresova povezanih sa formiranjem leda u vodenom rastvoru proteina [40], jer se većina proteina denaturiše na temperaturama znatno nižim od 0 °C [1].

2.3.1.1. Uticaj niske temperature na denaturaciju proteina

Do denaturacije proteina na niskim temperaturama dolazi primarno zbog narušavanja faktora koji najviše doprinosi stabilnosti native strukture proteina, hidrofobnog efekta [41]. Eksperimentalno je utvrđeno da je voda koja okružuje hidrofobne strukture uređenija, jer formira klatrate oko istih. Ova uređenost vode termodinamički je nepovoljna zbog smanjene entropije vode. Zato hidrofobne strukture imaju tendenciju da se grupišu, smanjujući time kontaktnu površinu sa vodom, a time i uređenost strukture vode oko njih. Na taj način se prilikom uvijanja proteina povećava entropija vode [42] (slika 2.2). Međutim, na dovoljno niskoj temperaturi, smanjena je kinetička energija vode, te razlika u neuređenosti vode u rastvoru i oko hidrofobnih ostataka nije velika, zato na niskoj temperaturi neće biti favorizovano uvijeno, već denaturisano stanje proteina. Drugim rečima, doći će do kolapsa hidrofobnog efekta [41]. Sekundarno, denaturacija na niskim temperaturama dešava se i zbog narušavanja hidrofobnih interakcija unutar jezgra proteina. Uzevši u obzir da hidrofobne interakcije nastaju kao posledica hidrofobnog efekta (odnosno, da je njihovo nastajanje vođeno predominantno entropijski), usled kolapsa hidrofobnog efekta dolazi i do narušavanja hidrofobnih interakcija [6]. Iako slabe, hidrofobne interakcije su po broju dominantne

interakcije koje stabilizuju nativno stanje proteina [43]. Takođe, jačina ovih interakcija zavisi od temperature, smanjujući se sa snižavanjem temperature. Usled toga na dovoljno niskim temperaturama dolazi do razvijanja kompaktne proteinske strukture i izlaganja nepolarnih ostataka vodi [1]. Denaturaciju proteina na niskim temperaturama po pravilu prati promena obrasca sekundarnih struktura, odnosno porast sadržaja β -pločica [44, 45].

2.3.1.2. Uticaj formiranja leda na denaturaciju proteina

Zamrzavanje rastvora proteina indukuje nekoliko odvojenih efekata koji predstavljaju stres za strukturu proteina i time dovode do denaturacije. Najvažniji faktori odnose se na kolaps hidrofobnog efekta i destabilizaciju hidratacione ljuske proteina usled stvaranja kristala leda, ali i lokalno koncentrovanje supstanci prisutnih u rastvoru proteina koje može dovesti do diferencijalnog taloženja rastvoraka [46]. Usled formiranja leda u rastvoru proteina dolazi do lokalnog koncentrovanja i samog proteina, što može dovesti do agregiranja. U nekim slučajevima, nasuprot tome, sa porastom koncentracije proteina raste i stabilnost. Takav je slučaj, na primer, kod β -galaktozidaze, fosfofruktokinaze i laktat-dehidrogenaze [47]. Koncentracija elektrolita u neposrednoj okolini proteina koja može tokom zamrzavanja da se poveća i do 40 puta u slučaju soli kao što je natrijum-hlorid, može, takođe, da destabilizuje protein ili da vodi isoljavanju [47]. Selektivna precipitacija jedne od komponenti pufera može da izazove drastičnu promenu pH vrednosti za čak 4 ili više pH jedinica kao što je slučaj sa dinatrijum-fosfatom, za razliku od dikalijum-fosfata koji je mnogo rastvorljiviji u vodi (promena pH rastvora kalijum-fosfatnog pufera usled zamrzavanja iznosi samo oko 0,3 pH jedinice) [47].

Pokazano je da je jedan od faktora denaturacije na niskim temperaturama i interakcija proteina sa dodirnom površinom led-voda. Eksperimentalno je zapaženo da kada se maksimalno eliminišu drugi faktori koji dovode do denaturacije, a posmatra samo uticaj brzine zamrzavanja i odmrzavanja, najveću očuvanost strukture proteina obezbeđuje kombinacija sporog zamrzavanja (oko 1 °C/min) i brzog odmrzavanja (> 10 °C/min). Nameće se logično objašnjenje ovog eksperimentalnog zapažanja: ukoliko je brzina zamrzavanja prevelika, došlo bi do stvaranja velikog broja kristala leda na većem broju mesta u rastvoru što bi dalo zbirno mnogo veću kontaktnu površinu led-voda. Brzo

odmrzavanje, s druge strane, smanjuje mogućnost lokalne rekristalizacije, a time i oštećujući efekat kristala leda na proteine [46].

2.3.2. Značaj proučavanja stabilnosti proteina na niskim temperaturama

Temperaturna stabilnost, kako na visokim, tako i na niskim temperaturama, glavni je faktor koji ograničava korišćenje proteolitičkih enzima u industriji [3]. S tim u vezi, osim fundamentalnog značaja proučavanja stabilnosti proteina na niskim temperaturama, i mnogi praktični problemi upotrebe, transporta i skladištenja proteina na niskim temperaturama biće rešeni ako se mehanizam denaturacije do kraja razjasni. Razumevanje stabilnosti proteina na niskim temperaturama od izuzetne je važnosti i u proučavanju adaptacije živih bića na ekstremno niske temperature [4]. U farmaceutskoj industriji je priprema adekvatne proteinske formulacije koja će omogućiti povećanu stabilnost proteina od značaja, naročito kada se uzme u obzir da je sa razvojem tehnologije rekombinantne dezoksiribonukleinske kiseline (*engl.* Deoxyribonucleic Acid - DNA) sve veći broj proteinskih terapeutika ušao u komercijalnu primenu [48]. Osim toga, u rutinskoj laboratorijskoj praksi često se javlja potreba za dugoročnim čuvanjem proteina u rastvornom obliku. Pri tom, vek trajanja proteina u rastvoru duži je ukoliko se čuva u zamrznutom stanju. Za rastvore proteina, koji se koriste u različitim granama industrije, često se javlja potreba za skladištenjem mnogo većih zapremina. Problem nastupa kada se rastvor proteina više puta zamrzava i odmrzava jer tada dolazi do narušavanja native strukture proteina, a time i do pada aktivnosti. Zamrzavanje dovodi do denaturacije proteina i prilikom liofilizacije [47]. Svi navedeni problemi koji su usko vezani za stabilnost proteina na niskim temperaturama, ukazuju na veliki značaj proučavanja ovog fenomena, kao i pronalaženja načina za povećanje stabilnosti proteina na niskim temperaturama.

2.3.3. Eksperimentalno proučavanje stabilnosti proteina na niskim temperaturama

Proučavanje denaturacije proteina na niskim temperaturama od početka su pratili metodološki problemi, naročito kada se uzme u obzir da mnogi monomerni proteini ostaju stabilni do 0 °C, odnosno da do denaturacije dolazi na temperaturama ispod tačke zamrzavanja vode, što je dodatno komplikovalo iznalaženje testova za praćenje

inaktivacije i denaturacije u zamrznutim rastvorima [1]. Primenjeno je nekoliko različitih pristupa da bi se ova prepreka prevazišla. Jedan od ranijih pristupa bilo je destabilizovanje proteina u cilju pomeranja temperature denaturacije proteina ka vrednostima koje su iznad 0 °C. Naime, primenom dodatnog spoljašnjeg faktora, kao što je korišćenje denaturanata ili povišenog pritiska, kao i uvođenjem mutacija, denaturacija proteina omogućena je na temperaturama iznad 0 °C. Nedostatak ovakvog pristupa je u tome što se njime dobija informacija o stabilnosti proteina u okruženju koje je delimično nenativno ili je sama primarna struktura promenjena [49]. S druge strane, često je primenjivan i pristup dodavanja supstanci koje snižavaju tačku mržnjenja vodenog rastvora za nekoliko stepeni ili pristup formiranja emulzija u kojima se proteini nalaze u vidu micela u vodenom rastvoru, uz primenu superhlađenja, pri čemu se omogućava da u rastvoru proteina ne dođe do formiranja kristala leda [6]. I ovakvi pristupi imaju nedostatak, a to je da se ne može eliminisati uticaj dodatnih supstanci (aditiva) na stabilnost proteina [50]. Poslednjih godina denaturacija proteina na niskim temperaturama prati se i nuklearnom magnetnom rezonancijom (*engl.* Nuclear Magnetic Resonance - NMR). Zamrzavanje vodenog rastvora u kapilari sprečeno je i do -16 °C usled kapilarnih pojava dodatkom poliakrilamidnog gela, koji formira trodimenzionalni sistem pora (koje se mogu posmatrati kao mreža kapilara manjeg promera) [8, 51, 52]. Bez obzira što su ovako dobijeni dragoceni podaci o mehanizmu denaturacije proteina na niskim temperaturama, ova metodologija ne nailazi na rutinsku primenu, već je ograničena na proučavanje par model sistema: male jednodomenske rekombinantne proteine koji su obogaćeni retkim izotopima ugljenika i azota (C^{13} i N^{15}) [8]. Jedan od retkih primera model sistema proteina za proučavanje denaturacije na niskim temperaturama koji se denuriše iznad 0 °C je frataksin kvasca. Njegova karakteristika niske termodinamičke stabilnosti i denaturacije na dovoljno visokim temperaturama u hladnom regionu, postavila ga je na važno mesto kada je proučavanje denaturacije na niskim temperaturama u pitanju [49]. Upravo je na ovom proteinu nedvosmisleno pokazano da do denaturacije dolazi i na visokim i na niskim temperaturama, bez dodatnih destabilizujućih agenasa, već pri fiziološkim uslovima [9].

Jedan od pristupa koji omogućava proučavanje stabilnosti proteina na niskim temperaturama jeste izlaganje proteina uzastopnim ciklusima zamrzavanja/odmrzavanja. Ovaj pristup često se koristi u farmaceutskim studijama

stabilnosti proteinskih terapeutika u vodenim rastvorima. Prednost pristupa izlaganja proteina ciklusima zamrzavanja/odmrzavanja sastoji se u tome što se na taj način dobija kombinovani uticaj i niske temperature i stresova povezanih sa formiranjem leda na stabilnost proteina. Sa praktične tačke gledišta ovakav pristup je optimalan u slučaju komercijalnih proteina koji se koriste/čuvaju na temperaturama ispod nule jer daje sliku sveobuhvatne stabilnosti proteina u realnim uslovima [40, 53]. Međutim, kada je u pitanju fundamentalno istraživanje stabilnosti proteina na niskim temperaturama, postoji potreba za razdvajanjem uticaja samo niske temperature od drugih stresova [40].

Prva uspešna eksperimentalna zapažanja denaturacije proteina u fiziološkim rastvorima (bez denaturanata) uočena su 80-ih godina prošlog veka na model proteinima himotripsinogenu [1], laktat-dehidrogenazi [4], mioglobinu [1] i apomiglobinu [50]. Pokazano je da do denaturacije dolazi na temperaturama koje su značajno ispod nule, kao što su i ranije kalkulacije pretpostavljale. Ove studije su prvi put pokazale da do denaturacije proteina dolazi i samo usled snižavanja temperature (izvedene su u nezaleđenom fiziološkom rastvoru proteina superhlađenjem) [1]. Denaturacija globularnih proteina na niskim temperaturama detektovana je na velikom broju proteina, naročito na povišenom pritisku [1, 50, 54]. Jedan od proteina koji je najviše proučen u kontekstu denaturacije na niskim temperaturama je i ribonukleaza A, u čijem slučaju je prvi put primećeno, korišćenjem tehnike NMR, da konformacija denaturisanog proteina može imati rezidualne sekundarne strukture i da se dobijeno stanje razlikuje od stanja nakon denaturacije povišenom temperaturom [54]. U studiji koja je kao model sistem koristila stafilokoknu nukleazu primenom tehnika fluorescencije, infracrvene spektroskopije sa Furijeovom transformacijom (*engl.* Fourier Transform Infrared Spectroscopy - FT-IR) i tehnike rasejanja x-zraka (*engl.* Small Angle X-ray Scattering - SAXS) dokazana je takođe denaturacija na niskim temperaturama [54]. Kao model sistemi ranih studija denaturacije na niskim temperaturama potpomognutim visokim pritiskom, korišćeni su i lizozim, ubikvitin, proteini mleka (α -laktalbumin i β -laktoglobulin), kao i nekoliko multidomenskih proteina u kojima je u većini slučajeva detektovano pogrešno uvijeno stanje sa prisutnim sekundarnim strukturama [54]. Veliki broj studija pokazao je da u proteinima denaturisanim niskom temperaturom dolazi do porasta sadržaja β -pločice u poređenju sa nativnim proteinima [44, 45]. Poznato je, takođe, da β -pločice predstavljaju gradivne

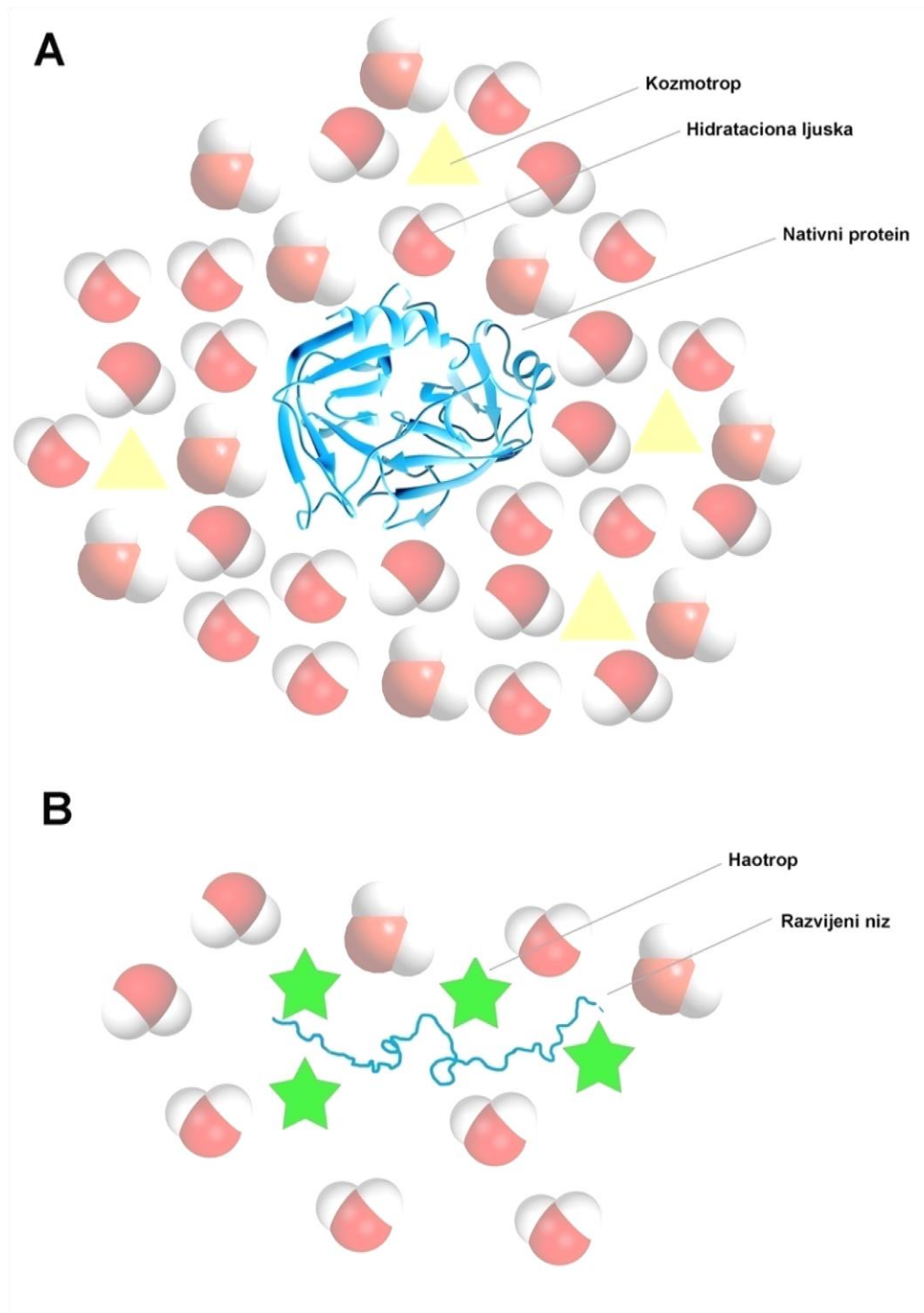
elemente agregata [55], te nije iznenađujuće detektovanje agregiranja koje u nekim slučajevima prati denaturaciju proteina na niskim temperaturama [56].

2.3.4. Povećanje stabilnosti proteina na niskim temperaturama

Postoji više različitih mehanizama kojima se može postići povišena stabilnost proteina na niskim temperaturama. Pre svega, danas je dobro poznato da postoje supstance (krioprotektanti) čije prisustvo u rastvoru proteina dovodi do povećane termodinamičke stabilnosti proteina i to mehanizmom preferencijalnog potiskivanja sa površine proteina, odnosno izazivanjem preferencijalne hidratacije proteina. U pitanju je prethodno opisano kozmotropsko dejstvo na proteine na sniženoj temperaturi. U ovakve klase jedinjenja spadaju poliol, polimeri, šećeri, aminokiseline, kao i stabilizujuće soli sa kozmotropskim efektom [48].

2.3.4.1. Krioprotektanti

Dodavanje različitih stabilizujućih organskih supstanci u rastvore labilnih enzima, uobičajena je praksa zaštite enzima tokom skladištenja [57]. Proučavanjem uticaja pojedinih malih molekula na stabilnost proteina u vodenom rastvoru na niskim temperaturama, došlo se do zaključka da strukturno različite klase molekula dovode do stabilizacije proteina, uključujući poliole/polimere, šećere, aminokiseline, metilamine, pa čak i neke soli [42, 57]. To je otežavalo pronalaženje mehanizma kojim sve te klase molekula dovode do istog efekta. Pokazano je, međutim, da je u pitanju univerzalni fenomen za sve navedene klase jedinjenja. Naime, ova jedinjenja su preferencijalno potisnuta sa površine proteina, što znači da stabilizuju protein ne interagujući s njim. Uticaj različitih rastvorenih supstanci na stabilnost proteina će, prema tome, zavisiti od toga da li dato jedinjenje interaguje sa proteinom ili ne. Preferencijalno potiskivanje će dovesti do stabilizovanja proteina (kozmotropni efekat), dok će preferencijalno vezivanje dovesti do destabilizovanja (haotropni efekat) (slika 2.6).



Slika 2.6. Šematski prikaz: A) stabilizujućeg efekta kozmotropa; B) destabilizujućeg efekta haotropa na strukturu proteina.

Dobro poznati primeri denaturanata, guanidin-hidrohlorid i urea, preferencijalno se vezuju za protein i time dovode do njegovog destabilizovanja. Postoje i izuzeci iz ove klasifikacije, jer je za neka jedinjenja primećeno da na niskim temperaturama imaju

stabilizujući efekat, dok na povišenim temperaturama imaju destabilizujući efekat na proteine. Primeri takvih molekula su polietilen-glikol (PEG), dimetilsulfoksid (DMSO), etanol i etilen-glikol. Ovakav paradoks proizilazi iz činjenice da hidrofobne interakcije ovih molekula sa proteinima zavise od temperature [42].

2.3.4.2. Mehanizam preferencijalnog potiskivanja

U studiji koju su sprovedi Timašev i saradnici prvi put je opisan mehanizam stabilizacije proteina krioprotektantima u vodenom rastvoru. U ovoj studiji pokazano je da je u neposrednoj blizini površine proteina koncentracija stabilizujućeg krioprotektanta veoma niska, odnosno da je koncentracija krioprotektanta bliska nuli, u odnosu na koncentraciju u okolnom rastvoru. Otuda je ovakav efekat pojedinih molekulskih klasa dobio opis „preferencijalna potisnutost sa površine proteina“. Površina proteina je stoga okružena predominantno vodom, te se kaže da je protein „preferencijalno hidratisan“. Ovakvo stanje je termodinamički nepovoljno jer podrazumeva visok hemijski potencijal i krioprotektanta i proteina. Međutim, kada bi došlo do razvijanja proteina, zauzelo bi se termodinamički nepovoljnije stanje jer bi kontakna površina između molekula krioprotektanta i proteina postala veća, odnosno njihovi hemijski potencijali bi bili još viši. Stoga sledi da je usled prisustva krioprotektanata koji su potisnuti sa površine proteina, razlika u slobodnoj energiji između nativnog i denaturisanog stanja još veća, odnosno protein je termodinamički stabilniji [48]. Kasnije je pokazano da isti mehanizam stabilizacije važi i tokom zamrzavanja/odmrzavanja u mikrosredinama vodenih rastvora proteina okruženih ledom koji se formira/otapa [58].

2.4. Model sistemi - proteaze

Proteolitički enzimi igraju fundamentalnu ulogu u mnogim fiziološkim procesima, od degradacije i sazrevanja proteina do specifičnijih regulatornih funkcija. Stoga karakterizacija proteolitičkih enzima pomaže u boljem razumevanju njihove uloge u fiziološkim procesima i otvara nove mogućnosti u komercijalnoj primeni istih. Proteaze koje predstavljaju enzime sa najširoom primenom u industriji hrane, farmaceutskoj industriji i industriji detergenata čine oko 60% ukupne svetske proizvodnje enzima [59, 60].

Proteaze (peptidaze ili proteinaze) predstavljaju enzime koji katalizuju hidrolizu peptidne veze. Prema mestu hidrolize peptidnog lanca mogu se klasifikovati kao endopeptidaze ili egzopeptidaze [61]. Egzopeptidaze hidrolizuju peptidne veze na N- ili C-terminusu polipeptidnog lanca. S druge strane, endopeptidaze, koje hidrolizuju peptidne veze u unutrašnjosti lanca [59], dalje se klasifikuju na osnovu reaktivnih grupa u aktivnom mestu koje učestvuju u katalizi i to na cistein-, serin-, aspartat- i metaloproteaze. Postoje i proteaze čiji reakcioni mehanizam nije u potpunosti razjašnjen i one su svrstane u posebnu grupu [61]. Proteaze obično sadrže autoinhibitorni prodomen koji mora biti uklonjen da bi se aktivirao enzim [62]. Takva neaktivna forma proteaze naziva se zimogen. Proregion je esencijalan za adekvatno uvijanje proteina i procesovanje, a deluje kao inhibitor proteina visokog afiniteta [61]. Aktivnost mnogih proteaza takođe zavisi i od pH, odnosno ćelijskog kompartmenta u kom su lokalizovani ili od prisustva endogenih proteaznih inhibitora ili aktivatora [62].

2.4.1. Lateks smokve (*Ficus carica*)

Smokva (*F. carica*) je biljka iz roda *Ficus* koji obuhvata preko 1800 vrsta što ga čini jednim od najvećih rodova u familiji Moraceae [63]. Ova vrsta gaji se vekovima unazad u tropskim i suptropskim krajevima zbog upotrebe u ishrani i tradicionalnoj medicini [64]. U istraživačkom smislu interesantna je zbog svog raznovrsnog sastava koji obuhvata različita organska jedinjenja, terpene, alergene, prirodnu gumu i proteolitičke enzime lateksa [65, 66].

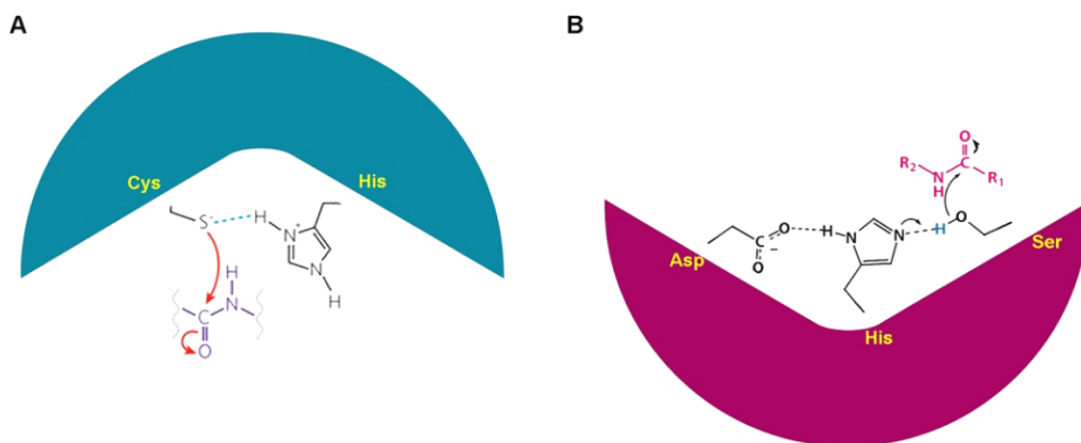
Lateks predstavlja citoplazmatsku tečnost posebne vrste biljnih ćelija, specijalizovanih sekretornih ćelija koje su poznate pod nazivom laticiferi (mlečne ćelije) [66]. Ove ćelije, usled delimične hidrolize ćelijskih zidova, formiraju mrežu cevčica ili paracirkulatorni sistem koji se pruža kroz biljku. Kada se ove cevi oštete usled povrede biljke, lateks ističe sa mesta povrede, koaguliše u kontaktu sa vazduhom i time sprečava invaziju patogena. U tome se ogleda mehanička zaštitna uloga lateksa koju duguje prevashodno kaučuku. Kaučuk (*cis*-1,4-poliizopren) smatra se sekundarnim metabolitom biljaka i sugerisano je da ima protektivnu ulogu u slučaju povrede tkiva [67]. Oko 4% lateksa smokve čini kaučuk mase 190 kDa [65]. Poznato je da lateks sadrži mnoge hidrolitičke enzime zadužene za odbranu biljke od insekata i mikroorganizama. U tome se ogleda biohemijska zaštitna uloga lateksa. Mlađi delovi drveta smokve (mladi izdanci, pupoljci

i listovi) sadrže najveće količine lateksa, jer lateks predstavlja njihov jedini mehanizam odbrane. Stariji delovi biljke imaju dodatne odbrambene strategije kao što je visoka koncentracija polifenola i čvršća i deblja kora, tako da sadrže mnogo manje lateksa [68]. Proteolitička frakcija lateksa roda *Ficus* predominantno sadrži cistein-proteaze, ali prisutne su i druge klase proteaza kao što su aspartat- [69] i serin-proteaze [63].

2.4.2. Cistein-proteaze

Cistein-proteaze, takođe poznate kao tiol-proteaze, široko su zastupljene među živim organizmima, i u prokariotama, i u eukariotama [70]. Do sada je otkriveno oko 20 familija cistein-proteaza [59]. Cistein- i serin-proteaze katalitički su veoma različite od aspartat- i metaloproteaza i to u tome što je nukleofil katalitičkog mesta deo aminokiseline, dok je kod druge dve grupe u pitanju aktivirani molekul vode. Katalitički mehanizam cistein-proteaza podrazumeva cisteinske grupe u aktivnom mestu [70]. Međutim, smatra se da katalitička aktivnost ovih enzima zavisi od katalitičke dijade koja se sastoji od cisteina i histidina (slika 2.7A), odnosno trijade cisteina, histidina i karboksilne grupe asparaginske kiseline [71]. Usled spontane oksidacije cisteinskog ostatka u aktivnom centru, cistein-proteaze su maksimalno aktivne u prisustvu blagih redukujućih agenasa kao što je cistein, ditiotreitol i β -merkaptotanol [72]. Na osnovu specifičnosti svrstane su u četiri grupe: proteaze slične papainu, proteaze slične tripsinu, proteaze specifične za glutaminsku kiselinu i druge koje ne pripadaju ni jednoj od prethodnih grupa [59].

Cistein-proteaze imaju pH optimum najčešće oko 7 iako neke od njih pokazuju maksimalnu aktivnost u kiseloj sredini. Podložne su inhibiciji reagensima koji alkiluju slobodnu tiolnu grupu cisteinskog ostatka kao što je N-[N-(L-3-transkarboksiiran-2-karbonil)-L-Leucil]-agmatin (E-64) i jodoacetamid (IAA), ali su rezistentne na metal helirajuće agense [59].



Slika 2.7. Šematski prikaz aktivnog mesta: A) cistein-proteaza; B) serin-proteaza.

Cistein-proteaze katalizuju hidrolizu dvostrukom izmenom uključujući opšte kiselo-bazno formiranje i hidrolizu acil-tiol intermedijera. Ovaj mehanizam je veoma sličan mehanizmu serin-proteaza. Još davne 1958. godine konstatovano je da je sulfhidrilna grupa acilovana za vreme reakcije između enzima i supstrata [59]. Cistein-proteaze zapravo katalizuju hidrolizu peptidne veze uz pomoć tiolatnog anjona (S^-). Ako je tiolatni anjon oksidovan do disulfida ili sulfinske kiseline enzim je reverzibilno ili čak ireverzibilno inaktiviran. Katalitička aktivnost se, stoga, održava dodavanjem tiolnog agensa kao što je cistein, ditiotreitol i β -merkaptetoanol [73, 74].

Biljni enzim, papain, iz lateksa biljne vrste *Carica papaya*, najintenzivnije je proučavan član među cistein-proteazama [61]. Biljni enzimi slični papainu sintetišu se u formi zimogena i karakteriše ih mala molekulska masa, široka supstratna specifičnost koju pokazuju *in vitro*, osetljivost na inhibitore cistein-proteaza i potreba za redukujućim agensima za potpunu aktivnost *in vitro* [70].

2.4.2.1. Papain

Papain (EC 3.4.22.2) je cistein-proteaza izolovana iz stabla (lateksa) ili ploda biljke papaja (*Carica papaya*) [75]. Veoma je dobro okarakterisan u naučnoj literaturi i odlikuje se širokom supstratnom specifičnošću i komercijalnom primenom. Za papain je pokazano da se prilikom izolovanja dobija inaktivna forma u kojoj je cisteinski ostatak

aktivnog mesta blokirano disulfidnom vezom sa drugim ostatkom cisteina. Aktivacija je moguća intermolekulskom razmenom disulfida sa tiolnim reagensima ili drugim redukujućim agensima [61]. Preprotein se sastoji od 345 aminokiselina i sintetizuje se u formi zimogena [76]. Nakon isecanja aktivacionog peptida zreli enzim sastoji se od 212 aminokiselinskih ostataka koji su organizovani u dva domena. Sekundarna struktura N-terminalnog domena prevashodno je α -helikoidna, dok u C-terminalnom domenu dominira antiparalelna β -pločica [77] (slika 2.8). Na osnovu eksperimentalno određene trodimenzionalne strukture papaina metodom difrakcije X-zraka, određeno je da nativni papain sadrži 42% neuređene strukture, dok je sadržaj α -heliksa i β -pločice sličan (26, odnosno 25%).



Slika 2.8. Trodimenzionalna struktura papaina. PDB kod 1bp4. Slika je kreirana korišćenjem programa UCSF Chimera verzija 1.11 (San Francisco, SAD). <http://www.rbvi.ucsf.edu/chimera>.

Za papain, kao i druge proteaze slične papainu, pokazano je da predstavljaju kinetički stabilne proteine. Kinetički stabilni proteini odlikuju se visokom barijerom aktivacione energije koja razdvaja nativnu konformaciju od prelaznog stanja na putu ka denaturaciji, bivajući time zaključani u nativnoj konformaciji. Kinetičku stabilnost treba razlikovati od termodinamičke stabilnosti. Pretpostavlja se da je kinetička stabilnost prisutna kod velikog broja proteina i da predstavlja regulatorni mehanizam ciklusa proteina *in vivo*. Naime, ovi proteini najčešće u svom denaturisanom/pogrešno uvijenom stanju pokazuju izraženu tendenciju za formiranje agregata (amiloidnih fibrila) koji su odgovorni za mnoga oboljenja. Samim tim smatra se da su strukturno evoluirali tako da su kinetički

inertni, što im omogućava da ostanu u svojoj nativnoj konformaciji i u uslovima koji su daleko od fizioloških [78]. Za ovakve proteine, između ostalih i za papain, pokazano je da je kinetička inertnost povezana sa rezistencijom na detergente kao što je natrijum-dodecilsulfat (*engl.* Sodium Dodecyl Sulfate - SDS). Proteaze slične papainu i drugi kinetički inertni proteini pokazuju neuobičajenu mobilnost u natrijum-dodecilsulfat poliakrilamidnoj gel elektroforezi (SDS-PAGE). Sporija migracija ovih proteina na nižoj temperaturi od 95 °C objašnjava se upravo kinetičkom stabilnošću native strukture za koju SDS ne može stehiometrijski da se veže [78]. Na visokoj temperaturi mobilnost kinetički stabilnih proteina drastično se menja.

Papain predstavlja važan komercijalni enzim. Široki spektar komercijalne primene papaina ogleda se u korišćenju u industriji hrane i pića [75, 79], stomatologiji [80], laboratorijskim i kliničkim preparacijama [81, 82], kozmetici [83] i industriji detergenata [84]. Ovako raznovrstan spektar primene papaina moguć je zahvaljujući, pored visoke aktivnosti i široke supstratne specifičnosti, visokoj stabilnosti na povišenim temperaturama (sa T_m vrednošću (vrednost temperature na kojoj je 50% proteina u nativnoj konformaciji, *engl.* melting temperature) od 83 ± 1 °C) [3]. Međutim, stabilnost na niskim temperaturama (naročito tokom zamrzavanja i odmrzavanja) nikada ranije nije proučavana.

2.4.2.2. Ficin

Ficin (EC 3.4.22.3), za koga se dugo smatralo da je jedan enzim sa eventualnim izoformama, predstavlja smešu različitih formi i izoformi koje su okarakterisane kao sulfhidrilni enzimi, jer sadrže cisteinske ostatke u aktivnom mestu koji su esencijalni za njihovu aktivnost [85]. Najviše proučavani ficini su cistein-proteaze lateksa vrste *Ficus glabrata* i *Ficus carica* [63], ali je i pored toga veoma malo informacija dostupno o njihovim fizičko-hemijskim karakteristikama. Ono što je do sada otkriveno je da ficin deli veliki broj karakteristika sa papainom, kao što je supstratna specifičnost, esterazna i transpeptidazna aktivnost i aktiviranje redukujućim agensima [73, 86]. Stoga su ove cistein-proteaze svrstane u papainsku familiju (subfamilija C1A po Merops bazi podataka) [87].

Ficini su proteini visoke proteolitičke aktivnosti u širokom opsegu temperatura i pH vrednosti. U pitanju su bazni proteini koji se mogu frakcionisati na katjonskom izmenjivaču [63, 85]. Još 1964. godine prijavljeno je razdvajanje nekoliko formi ficina i od tada je publikovano još nekoliko radova na temu postojanja više formi ficina u lateksu *F. carica* [73, 85]. Tačan broj različitih formi ficina nije poznat i osim iz studija Devaraja i saradnika nema dostupnih fizičko-hemijskih i/ili strukturnih studija prečišćenih formi ficina [63]. Oni su pokazali da su u pitanju proteini koji se sastoje od jednog polipeptidnog lanca, molekulske mase između 23 i 24 kDa, da su aktivni na pH 7,0 i da do potpunog inaktiviranja dolazi na pH 3,0 [63].

Analiza masenom spektrometrijom u studiji Azarkana i saradnika pokazala je postojanje 4 različite cistein-proteaze označene kao A, B, C i D u lateksu *F. carica*, dok D1 i D2 predstavljaju verovatno dve izoforme. Precizno su određene i molekulske mase svake forme (od $23848,10 \pm 27,29$ Da do $24454,80 \pm 7,54$ Da). U ovoj studiji potvrđeno je prisustvo cisteina u katalitičkom mestu. Naime, enzimska aktivnost sve četiri proteaze potpuno je inhibirana jodoacetamidom, E-64, kao i inhibitorom S-metil metantiosulfonat (MMTS), koji predstavljaju specifične inhibitore cistein-proteaza u testu sa sintetičkim supstratom N α -benzoil-DL-arginin 4-nitroanilid-hidrohlorid (BAPNA). Tozil lizil hlorometan hidrohlorid (TLCK) i tozil fenilalanil hlorometil keton (TPCK) predstavljaju inhibitore proteaza sličnih tripsinu, odnosno himotripsinu, za koje je takođe nađeno da su efikasni u inhibiciji ovih enzima. Međutim, za ove inhibitore je poznato da mogu da inhibiraju i cistein-proteaze kao što je papain, bromelain i klostripain. Osim toga, strukturna analiza difrakcijom X-zraka kompleksa papain-TPCK pokazala je da je katalitički ostatak cisteina ireverzibilno alkilovan. Pefablok SC, specifični inhibitor serin-proteaza, pepstatin A, specifični inhibitor aspartat-proteaza i EDTA (etilendiamin tetraacetat), inhibitor metaloproteaza, ne utiču na enzimsku aktivnost ficina kada se BAPNA koristi kao supstrat [88].

U nedostatku podataka o strukturi proteina ficina (sekvenca, kristalna struktura) upotrebom specifičnih inhibitora zaključeno je da je okolina esencijalnog cisteinskog ostatka ficina iz lateksa smokve slična aktivnom centru papaina i u blizini je imidazolove grupe histidinskog ostatka [72].

Za nekoliko izolovanih formi ficina određena je sekundarna struktura metodom cirkularnog dihroizma (CD), pri čemu je nađeno da u strukturi ficina dominira neuređena struktura (35-40%). Procenat α -heliksa je 19-24%, dok β -pločice zauzimaju 22-26%, a zavijutak 18-19% [88].

U prethodnim istraživanjima pokazano je da ficinske (izo)forme poseduju široku supstratnu specifičnost koja uključuje i hidrolizu kazeina i sintetičkog supstrata BAPNA koji su najčešće korišćeni supstrati za praćenje aktivnosti ficina [63, 88].

2.4.3. Serin-proteaze

Serin-proteaze su jedna od najvećih grupa proteolitičkih enzima i učestvuju u brojnim regulatornim procesima. Pokazana je uloga serin-proteaza u različitim ćelijskim mehanizmima kao što je signalna transdukcija, diferencijacija, hipersenzitivnost, digestija hrane i druge. Međutim, smatra se da postoji još mnogo funkcija i regulatornih uloga serin-proteaza koje još uvek nisu otkrivene [60].

Katalitički mehanizam serin-proteaza zasniva se na postojanju reaktivnog ostatka serina u aktivnom mestu ovih enzima. Reakcioni mehanizam preciznije obuhvata katalitičku trijadu (serin-aspartat-histidin) (slika 2.7B). Neki od najbolje proučenih enzima koji pripadaju ovoj familiji proteaza čine tripsin, himotripsin, elastaza, suptilizin, proteaze koje učestvuju u koagulaciji krvi i drugi. Ovi enzimi aktivni su na neutralnom i alkalnom pH, sa optimumom između pH 7,0 i 11,0 i pokazuju širok spektar supstratne specifičnosti uključujući esterolitičku i amidaznu aktivnost. Karakterišu se ireverzibilnom inhibicijom inhibitorima fenilmetilsulfonil fluorid (PMSF) i TLCK [63].

2.4.3.1. *Tripsin*

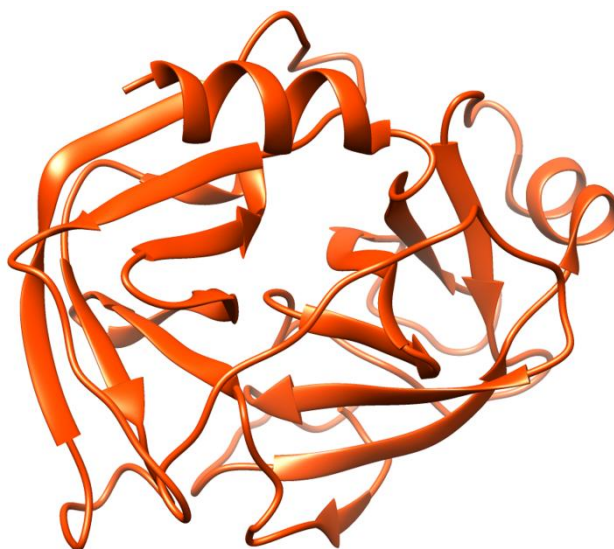
Tripsin je serin-proteaza sisara koja učestvuje u digestiji proteina hrane i sintetise se u ćelijama pankreasa u formi zimogena [89]. Zreli protein sastavljen je od jednog polipeptidnog lanca sa 223 aminokiseline i 6 disulfidnih veza [90]. Tripsin je visokospecifična proteaza koja katalizuje hidrolizu peptidne veze iza pozitivno naelektrisanih aminokiselina lizina (K) i arginina (R) [91]. Optimalna pH vrednost za aktivnost tripsina nalazi se u baznoj oblasti (oko 8) [92]. Kako tripsin u svojoj sekvenci sadrži 14 K i dva R, on ima izraženu sposobnost autoproteolize koja je detektovana u

literaturi. Određeno je da 1 mg/mL tripsina na svojoj optimalnoj pH vrednosti kompletno autoproteolizuje tokom 2h na sobnoj temperaturi [90]. Da bi se redukovala autoproteolitička aktivnost tripsina za sekvenciranje, često je neophodna hemijska modifikacija lizinskih ostataka tripsina reduktivnim metilovanjem koja rezultuje redukcijom brzine autoproteolize za 70% [93].

Poznato je da proteaze gube aktivnost ukoliko se čuvaju u rastvornom obliku na niskim temperaturama (zamrzavanje), te stoga proizvođači ovih enzima preporučuju njihovo skladištenje u liofilizovanoj formi. Uprkos tome, laboratorijska praksa svedoči da se često javlja potreba za skladištenjem tripsina za proteomiku i tripsina za ćelijske kulture u rastvornoj formi. Proizvođači ovih preparata preporučuju rastvaranje tripsina za proteomiku/sekvenciranje u kiselim uslovima (hlorovodoničnoj kiselini (HCl) ili sirćetnoj kiselini) da bi se sprečila intenzivna autoproteoliza [89]. Praksa je, međutim, pokazala da je takvo skladištenje ograničeno na veoma mali broj ciklusa zamrzavanja/odmrzavanja.

Poznato je da tripsin može biti reverzibilno inaktiviran na kiselim pH vrednostima [89]. U literaturi se mogu pronaći detaljni podaci o denaturaciji tripsina u kiseloj sredini [94] i na povišenom pritisku [95], pri čemu je pokazano da dolazi do denaturacije uz formiranje stabilnih intermedijera (stopljenih globula).

U sekundarnoj strukturi tripsina preovladava antiparalelna β -pločica, ali je prisutan i α -heliks koji su podjednako raspoređeni u dva domena tripsina [96] (slika 2.9). Na osnovu podataka o kristalnoj strukturi tripsina iz Proteinske banke podataka (*engl.* Protein Data Bank - PDB), određeno je da sadržaj β -pločica iznosi 46,2%, dok je procenat α -heliksa samo 8,1%. Neuređene strukture zauzimaju 33,6%, a zavijutak 12,1%.

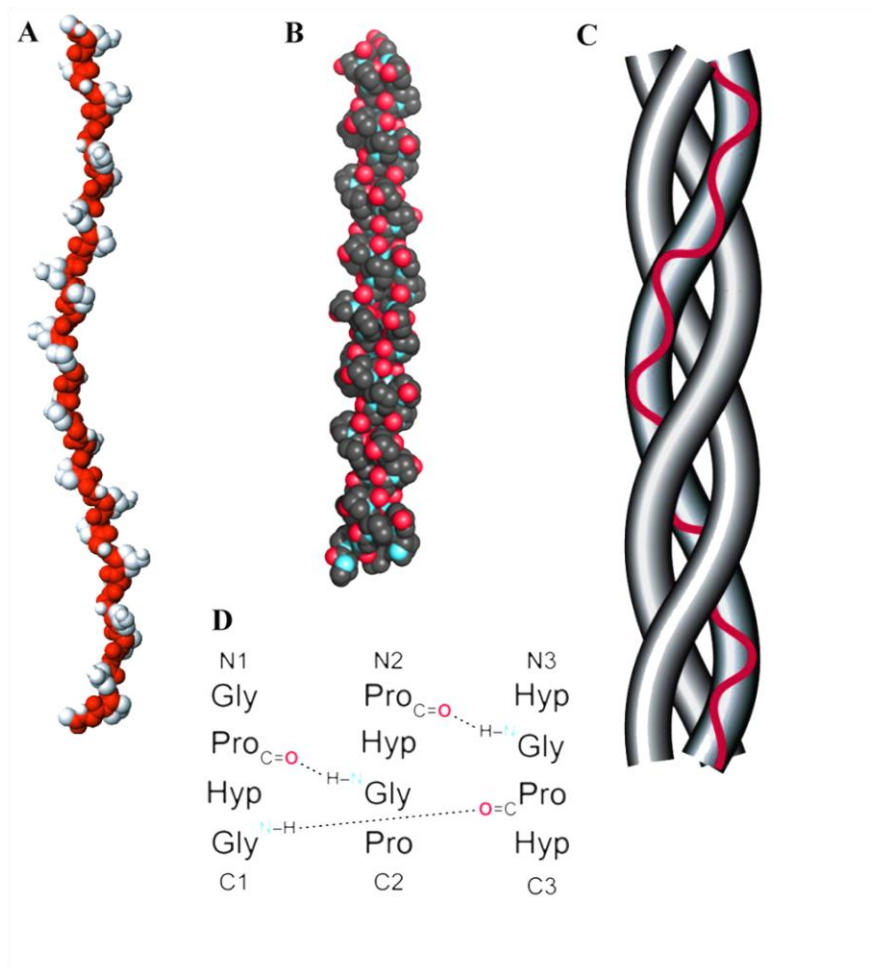


Slika 2.9. Trodimenzionalna struktura tripsina. PDB kod 1aq7. Slika je kreirana korišćenjem programa UCSF Chimera verzija 1.11 (San Francisco, SAD). <http://www.rbvi.ucsf.edu/chimera>.

Trypsin predstavlja enzim koji je dobro proučen i opisan u naučnoj literaturi i koji se odlikuje širokom komercijalnom primenom. Neke od važnih oblasti primene tripsina podrazumevaju digestiju proteina na laboratorijskom i industrijskom nivou [97], procesovanje adherentnih ćelijskih kultura [98], a u novije vreme tripsin se intenzivno koristi kao važno enzimsko sredstvo za identifikaciju i sekvenciranje proteina [99].

2.4.4. Kolagenaze

Kolagenazama se smatraju enzimi koji katalizuju hidrolizu nativnog kolagena u regionu trostrukog heliksa. Molekul kolagena izgrađen je od tri polipeptidna lanca koji se nazivaju α -lanci sa karakterističnom ponavljajućom sekvencom: Gly-X-Y, gde X u najvećem procentu čini ostatak prolina, dok je Y najčešće ostatak hidroksiprolina (slika 2.10), i svaki lanac je obično dug preko 1000 aminokiselinskih ostataka [100]. Kod nekih tipova kolagena sva tri lanca su identična, dok drugi sadrže dva ili čak sva tri različita lanca ($\alpha 1$, $\alpha 2$ i $\alpha 3$). Kolagen tipa I sadrži dva $\alpha 1$ lanca i jedan $\alpha 2$ lanac [101]. Iz triplet ponavljajuće primarne strukture proizilazi kvaternarna struktura karakteristična za nativni kolagen: tri leva heliksa poliprolinskog tipa (α -lanci) uvijena u desni trostruki heliks [101].



Slika 2.10. Prikaz modela: A) levog α -lanca i B) desnog trostrukog heliksa; C) šematski prikaz trostrukog heliksa kolagena; D) prikaz interlančanih vodoničnih veza u trostrukom heliksu kolagena.

Upravo složena, kolagenu svojstvena kvaternarna struktura, ne dozvoljava svim enzimima sa želatinolitičkom aktivnošću, da hidrolizuju i nativni kolagen. Naprotiv, veoma mali broj enzima koji hidrolizuju želatin može da hidrolizuje i nativni kolagen. Takođe, mnoge proteaze mogu da katalizuju hidrolizu globularne glave prisutne na krajevima molekula kolagena, ali manji je broj proteaza koje mogu da akomodiraju trostruki heliks u svoje aktivno mesto i hidrolizuju ga. Ti malobrojni enzimi smatraju se kolagenazama [102]. U novijoj literaturi pokazano je da jedina komercijalna kolagenaza, metalokolagenaza iz bakterijskog soja *Clostridium histolyticum* zapravo jednim svojim domenom raspliće trostruki heliks kolagena, a potom katalizuje hidrolizu u okviru pojedinačnih lanaca kolagena drugim domenom [103].

Animalne kolagenaze čine dominantno metalokolagenaze (matriks metalopeptidaze), ali postoje i serin-kolagenaze i cistein-kolagenaze [104]. Ostale kolagenaze (u koje spadaju mikrobne i biljne) na osnovu strukture aktivnog mesta mogu se klasifikovati na: metalo, cistein- i serin-kolagenaze [105]. Metalokolagenaze u aktivnom mestu sadrže jon cinka. Za njihovu stabilnost i optimalnu aktivnost obično je neophodan i kalcijum. U literaturi su proučavane i opisane metalokolagenaze iz različitih tkiva sisara (gde igraju ulogu u remodelovanju ekstracelularnog matriksa) [104], kao i iz bakterija, kao što je *Clostridium histolyticum* [106].

Kolagenolitičke serin-proteaze (serin-kolagenaze) otkrivene su u mnogim vrstama kraba i riba. Njihova fiziološka uloga u ovim organizmima zasniva se na digestiji hrane [107]. Sve do sada otkrivene serin-kolagenaze ispoljavaju širu supstratnu specifičnost, za razliku od metalokolagenaza sisara koje su isključivo specifične za kolagen [108, 109].

Metalokolagenaze sisara imaju usku supstratnu specifičnost, često sa tačno određenim mestom hidrolize u okviru same sekvence kolagena [104], dok se do sada opisane biljne i mikrobne kolagenaze odlikuju širokom supstratnom specifičnošću, budući da osim sposobnosti hidrolize nativnog kolagena pokazuju i opštu proteolitičku aktivnost [105, 106].

Primena kolagenolitičkih enzima veoma je raznovrsna i obuhvata veći broj oblasti, kao što su biotehnologija, medicina, farmakologija i industrija. Kolagenaze se primenjuju za izolovanje i kultivaciju sisarskih ćelija [110], kao i za prečišćavanje ćelija krvi u medicinskoj dijagnostici [111]. U industriji hrane, kolagenaze se koriste za preradu mesa [112]. Osim toga, primenjuju se u preradi kože i krzna [113]. Kolagenaze, takođe, igraju važnu ulogu u uspešnom presađivanju organa [114, 115], uklanjanju ožiljaka [116, 117], kao i tretmanu opekotina [118]. Neke od primena kolagenaze u terapijske svrhe podrazumevaju zaceljivanje rana [118], kao i lečenje različitih tipova destruktivnih fibroza, kao što je ciroza jetre [119]. I sami peptidi koji su proizvodi dejstva kolagenaza na kolagen ili želatin, nalaze primenu u kozmetici, imunoterapiji, ishrani. Oni se koriste i kao agensi za tretman osteoporoze, hipertenzije i čira na želucu [120]. Uzimajući u obzir veliki potencijal primene kolagenaza i potrebe za njihovim izolovanjem, od izuzetne je važnosti otkrivanje i karakterizacija novih kolagenolitičkih enzima. U odnosu na kolagenaze životinjskog i bakterijskog porekla, biljne kolagenaze

su mnogo manje proučene. Jedina do danas opisana biljna kolagenaza je kolagenolitička cistein-proteaza đumbira. U pitanju je enzim sličan papainu sa širokom supstratnom specifičnošću [105].

3. CILJEVI

Temperaturna stabilnost enzima koji imaju potencijal upotrebe u komercijalne svrhe (različite oblasti biotehnologije, medicine i nauke), jedan je od glavnih ograničavajućih faktora za njihovo korišćenje. Razumevanje preciznih strukturnih rearanžmana koje izaziva niska temperatura, pomoći će u definisanju optimalnih uslova pri kojima se može produžiti vek trajanja komercijalnih enzima, odnosno, pri kojima će oni biti stabilni.

Stoga, u okviru ovog istraživanja, formulisani su sledeći ciljevi:

a. Izolovanje enzima koji su predstavljali model sisteme ove studije

Kao model sistemi ove studije izabrana su dva enzima iz klase cistein-proteaza (papain i ficin) i dva enzima iz klase serin-proteaza (tripsin i kolagenaza), tako da je prvi cilj bilo dobijanje čistih/homogenih preparata ovih enzima (osim u slučaju tripsina (Sigma–Aldrich, Štajnhajm, Nemačka)):

- Prečišćavanje ficina i kolagenaze iz lateksa smokve (*F. carica*) i papaina iz komercijalnog preparata;
- Bazična biohemijska karakterizacija novootkrivene kolagenaze.

b. Optimizovanje metodologije za ispitivanje stabilnosti serin- i cistein-proteaza na niskim temperaturama

U cilju pronalaženja optimalne metodologije kojom se mogu pratiti promene aktivnosti i strukture proteaza koje se dešavaju kao posledica izlaganja niskoj temperaturi, ciljevi su bili sledeći:

- Ispitivanje stabilnosti papaina u širokom temperaturnom intervalu;
- Ispitivanje kinetičke stabilnosti (kriva termalne inaktivacije, rezistencija na detergent, ispitivanje tendencije za zauzimanje određenih sekundarnih struktura infracrvenom spektroskopijom).

c. Ispitivanje stabilnosti serin- i cistein-proteaza na niskim temperaturama

Glavni cilj ovog istraživanja jeste pronalaženje objašnjenja na nivou detaljnih strukturnih promena koja su u osnovi drastičnog gubitka aktivnosti kome proteaze podležu usled izlaganja niskim temperaturama.

- Ispitivanje promena aktivnosti proteaza na niskim temperaturama;
- Ispitivanje promena na nivou primarne strukture proteaza na niskim temperaturama (autoproteolize);
- Ispitivanje promena na nivou sekundarne/tercijarne strukture proteaza na niskim temperaturama;
- Ispitivanje agregiranja proteaza na niskim temperaturama.

d. Optimizovanje uslova za skladištenje tripsina za sekvenciranje u rastvornom obliku na niskoj temperaturi

Tripsin za sekvenciranje, proteaza od najvećeg komercijalnog značaja u ovoj studiji, izabran je kao model sistem za pronalaženje optimalnih uslova za čuvanje na niskoj temperaturi u rastvornom obliku.

- Poređenje promena aktivnosti i primarne, odnosno sekundarne/tercijarne strukture tripsina izlaganog niskim temperaturama u kiselj sredini (preporučenoj od strane proizvođača) i u blago alkalnoj sredini uz dodatak krioprotektivnih agenasa;
- Ispitivanje funkcionalnosti tripsina za sekvenciranje u predloženim optimalnim uslovima za čuvanje na niskim temperaturama.

4. MATERIJAL I METODE

4.1. Analitičke metode

4.1.1. Određivanje koncentracije ukupnih proteina

Koncentracija ukupnih proteina određena je Bredfordovom [121] i biuretskom metodom [122]. Standardne prave za određivanje koncentracije proteina konstruisane su korišćenjem različitih koncentracija rastvora goveđeg serum albumina (*engl.* Bovine Serum Albumin - BSA) (Sigma–Aldrich, Štajnhajm, Nemačka) kao standarda.

4.1.1.1. Bredfordova metoda za određivanje koncentracije proteina

Napravljen je rastvor boje u etanolu prema protokolu iz tabele 4.1, uz konstantno mešanje na magnetnoj mešalici do rastvaranja.

Tabela 4.1. Sastav Bredfordovog reagensa.

CBB G-250	250 mg
95% etanol	50 mL
96% fosforna kiselina	100 mL
Destilovana voda do	200 mL

Nakon rastvaranja dodata je kiselina i destilovana voda do potrebne zapremine. Za pripremu radnog reagensa ovako dobijeni Bredfordov reagens razblažen je pet puta destilovanom vodom i profiltriran kroz filter papir. Reagens je kao takav stabilan nekoliko meseci na sobnoj temperaturi.

Protokol:

U bunare mikrotitracione pločice sa ravnim dnom pipetira se po 5 μL uzoraka ili odgovarajućih standarda za makro metod, odnosno po 20 μL uzoraka ili odgovarajućih standarda za mikro metod. Uzorcima se doda po 200 μL radnog reagensa. Nakon inkubacije od 5 min na sobnoj temperaturi, meri se apsorbancija na 620 nm. Kao slepa proba se koristi 5 μL , odnosno 20 μL destilovane vode i 200 μL radnog reagensa.

4.1.1.1.1. Priprema različitih razblaženja BSA za konstruisanje standardne prave

Napravljen je polazni rastvor BSA koncentracije 1 mg/mL (tabela 4.2) koji je razblažen destilovanom vodom do finalnih koncentracija od 0,75 mg/mL, 0,50 mg/mL, 0,25 mg/mL, 0,10 mg/mL (tabela 4.3). Navedena razblaženja korišćena su za makro metod. Za mikro metod, napravljen je polazni rastvor BSA koncentracije 0,10 mg/mL (tabela 4.4) koji je razblažen destilovanom vodom do finalnih koncentracija od 0,075 mg/mL, 0,050 mg/mL, 0,025 mg/mL, 0,010 mg/mL (tabela 4.5).

Tabela 4.2. Polazni rastvor BSA (1,00 mg/mL) za Bredfordov makro metod.

BSA	5 mg
Destilovana voda do	5 mL

Tabela 4.3. Priprema standardnih rastvora BSA za Bredfordov makro metod.

Polazni rastvor BSA (μL) 1 mg/mL	Destilovana voda (μL)	Finalna koncentracija BSA (mg/mL)
1000	0	1,00
750	250	0,75
500	500	0,50
250	750	0,25
100	900	0,10

Tabela 4.4. Polazni rastvor BSA (0,10 mg/mL) za Bredfordov mikro metod.

BSA	1 mg
Destilovana voda do	10 mL

Tabela 4.5. Priprema standardnih rastvora BSA za Bredfordov mikro metod.

Polazni rastvor BSA (μL) 0,1 mg/mL	Destilovana voda (μL)	Finalna koncentracija BSA (mg/mL)
1000	0	0,100
750	250	0,075
500	500	0,050
250	750	0,025
100	900	0,010

Koncentracija proteina određena je sa standardne prave na osnovu jednačine prave koja je dobijena linearnom regresionom analizom kao apsorbancija u funkciji od koncentracije proteina.

4.1.1.2. Biuretska metoda za određivanje koncentracije proteina

Sastav i način pripreme biuretskog reagensa prikazan je u tabeli 4.6.

Tabela 4.6. Sastav biuretskog reagensa.

CuSO ₄ x 5H ₂ O	1,5 g
Na, K-tartarat	6,0 g
NaOH	30 g
Destilovana voda do	500 mL

*Ako se doda 1 g kalijum-jodida reagens je stabilan duži vremenski period

Protokol:

U 0,5 mL uzoraka (odnosno standardnih rastvora/slepe probe) dodaje se 2,5 mL reagensa. Posle 20-30 min meri se apsorbancija na 540 nm naspram slepe probe.

4.1.1.2.1. Priprema različitih razblaženja BSA za konstruisanje standardne prave

Standardni rastvori BSA pripremljeni su na način prikazan u tabeli 4.7.

Tabela 4.7. Priprema standardnih rastvora BSA.

Rastvor	Slepa proba	1 mg/mL	2 mg/mL	3 mg/mL	4 mg/mL	5 mg/mL
Destilovana voda (mL)	0,5	0,4	0,3	0,2	0,1	0,0
5 mg/mL BSA (mL)	0,0	0,1	0,2	0,3	0,4	0,5
Biuretski reagens (mL)	2,5	2,5	2,5	2,5	2,5	2,5

Koncentracija proteina određena je sa standardne prave na osnovu jednačine prave koja je dobijena linearnom regresionom analizom kao apsorbancija u funkciji od koncentracije proteina.

4.1.2. Elektroforetske tehnike

4.1.2.1. Natrijum-dodecil sulfat poliakrilamidna gel elektroforeza (SDS-PAGE)

4.1.2.1.1. Protokol za SDS-PAGE

Elektroforeza je rađena na Hoefer mighty small jedinici za elektroforezu po originalnoj Laemmli-jevoj proceduri [123]. Potrebni rastvori i sastav istih dat je u tabelama 4.8-4.18:

Tabela 4.8. Rastvor A: monomerni rastvor akrilamida i bisakrilamida (AA) (30% T¹, 2,7% C).

Akrilamid	58,4 g
Bisakrilamid	1,6 g
Destilovana voda do	200 mL

Tabela 4.9. Rastvor B: pufer za razdvajajući gel (1,5 M Tris² HCl, pH 8,8).

Tris	36,3 g
Destilovana voda do	200 mL
4 M HCl do	pH 8,8

Tabela 4.10. Rastvor C: pufer za koncentrujući gel (0,5 M Tris HCl, pH 6,8).

Tris	6,0 g
Destilovana voda do	100 mL
4 M HCl do	pH 6,8

Tabela 4.11. Rastvor D: 10% m/V natrijum-dodecil sulfat (SDS).

SDS	10 g
Destilovana voda do	100 mL

Tabela 4.12. Rastvor E: inicijator polimerizacije (10% m/V amonijum-persulfat (APS)).

APS	0,1 g
Destilovana voda do	1,0 mL

*Preporučuje se da se rastvor inicijatora polimerizacije napravi neposredno pre upotrebe.

¹T označava ukupan broj grama akrilamida i bisakrilamida u 100 mL rastvora, a C broj grama bisakrilamida u odnosu na zbirnu masu akrilamida i bisakrilamida. Obe vrednosti se izražavaju u procentima.

² Tris(hidroksimetil)aminometan (Tris)

Tabela 4.13. Rastvor F: rastvor za nadslojavanje gela pri polimerizaciji (n-butanol zasićen vodom).

n-butanol	100 mL
Destilovana voda	Do stabilnog donjeg sloja vode

*Rastvor pre upotrebe promućkati i ostaviti da se slojevi razdvoje; koristiti gornji sloj.

Tabela 4.14. Rastvor G: pufer za elektroforezu (0,025 M Tris, 0,192 M Gly, 0,1% SDS, pH 8,3).

Tris	3,0 g
Gly	14,4 g
SDS	1,0 g
Destilovana voda do	1000 mL

Tabela 4.15. Rastvor H: pufer za pripremu uzorka (PUZ) (5x koncentrat).

Reagens	Zapremina (mL)	Finalna koncentracija
0,5 M Tris pH 6,8	1,2	60 mM
98 % glicerol	5,0	25 %
10 % SDS	2,0	2 %
β-merkaptetanol*	0,5	14,4 mM
1 % bromfenol plavo	1,0	0,1 %
Destilovana voda do	10,0	

*Rastvor H za neredukujuću elektroforezu ne sadrži β-merkaptetanol, tako da se u ovom koraku dodaje destilovana voda.

Tabela 4.16. Rastvor I: rastvor boje (0,25% Coomassie Brilliant Blue (CBB), 50% metanol, 10% sirćetna kiselina).

CBB G- ili R-250	0,5 g
Metanol	100 mL
Sirćetna kiselina	10 mL
Destilovana voda do	200 mL

Tabela 4.17. Rastvor J: rastvor za fiksiranje (50% metanol, 10% sirćetna kiselina).

Metanol	500 mL
Sirćetna kiselina	100 mL
Destilovana voda do	1000 mL

Tabela 4.18. Rastvor K: rastvor za obezbojavanje (25% metanol, 5% sirćetna kiselina).

Metanol	250 mL
Sirćetna kiselina	50 mL
Destilovana voda do	1000 mL

Za rad je najčešće korišćen 12% gel za razdvajanje i 4% gel za koncentrovanje. Sastavi ova dva gela, kao i poliakrilamidnih gelova različitih koncentracija dati su u tabelama 4.19 i 4.20.

Tabela 4.19. Priprema poliakrilamidnog gela za razdvajanje različitih koncentracija (zapremine 10 mL).

Finalna koncentracija akrilamida u gelu (%)											
Rastvor	5	6	8	9	10	11	12	13	14	15	16
A (mL)	1,67	2,00	2,67	3,00	3,33	3,67	4,00	4,33	4,67	5,00	5,33
B (mL)	2,50	2,50	2,50	2,50	2,50	2,50	2,50	2,50	2,50	2,50	2,50
H ₂ O (ml)	5,67	5,33	4,67	4,33	4,00	3,67	3,33	3,00	2,67	2,33	2,00
TEMED ³ (μL)	5	5	5	5	5	5	5	5	5	5	5
D (mL)	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10
E (mL)	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05

Tabela 4.20. Priprema 4% poliakrilamidnog gela za koncentrovanje.

Finalna zapremina gela za koncentrovanje (mL)								
Rastvor	10	9	8	7	6	5	4	3
A (ml)	1,33	1,20	1,06	0,93	0,80	0,67	0,53	0,40
C (ml)	2,50	2,25	2,00	1,75	1,50	1,25	1,00	0,75
H ₂ O (ml)	6,00	5,40	4,80	4,20	3,60	3,00	2,40	1,80
TEMED (μL)	5,0	4,5	4,0	3,5	3,0	2,5	2,0	1,5
D (μl)	100	90	80	70	60	50	40	30
E (μl)	50	45	40	35	30	25	20	15

Postupak:

Prema podacima iz tabela 4.19 i 4.20 napravljeni su gelovi za elektroforezu (12% razdvajajući gel, odnosno 8% razdvajajući gel (u slučaju analize proizvoda digestije kolagena) i 4% koncentrujući gel) koji su pre dodatka odgovarajuće količine rastvora SDS-a (rastvor D) i APS-a (rastvor E) dezaerisani. Na samom kraju je dodat inicijator polimerizacije, a potom naliven donji gel (gel za razdvajanje) između staklenih ploča sistema za elektroforezu. Ovaj gel je nadslojen zasićenim rastvorom n-butanola (rastvor

³Tetrametiletilendiamin (TEMED)

F). Nakon polimerizacije donjeg gela, gel je ispran destilovanom vodom, a potom je naliven gornji gel (gel za koncentrovanje) i između staklenih ploča je postavljen češalj za formiranje bunara za nanošenje uzoraka. Ploče se nakon polimerizacije gela za koncentrovanje i nalivanja uzoraka u bunare urone u kadu sa puferom za elektroforezu (rastvor G).

Elektroforeza je rađena na konstantnoj struji od 25 mA po gelu.

Postupak detekcije proteinskih traka rastvorom boje CBB R-250 prikazan je u tabeli 4.21.

Tabela 4.21. Faze detekcije proteinskih traka.

Faza	Rastvor	Vreme (min)
Ispiranje	Destilovana voda	1
Fiksiranje	Rastvor J	15
Bojenje	Rastvor I	15
Obezbojavanje	Rastvor J	30
Obezbojavanje	Rastvor K	Do obezbojenja

4.1.2.1.2. Priprema uzoraka za elektroforezu

Pre nanošenja na gel, uzorci se mešaju sa pet puta koncentrovanim redukujućim puferom za uzorke (rastvor H) i inkubiraju 10 min na 95 °C. Koncentracija uzoraka proteina koja je nanošena na gel iznosila je približno 0,1 do 1 mg/mL (po Bredfordovoj ili biuretskoj metodi) (odeljak 4.1.1).

4.1.2.1.3. Određivanje molekulske mase

Molekulska masa proteina određena je poređenjem sa standardnim markerima molekulske mase (14,4-116 kDa) (Thermo Fisher Scientific, Rokford, IL, SAD). Kalibraciona kriva formirana je kao zavisnost pređenog puta traka standardnih markera molekulske mase od logaritma njihove molekulske mase.

4.1.2.2. Zimogramska detekcija proteina

U cilju zimogramske detekcije proteina, rađena je želatinska elektroforeza i to u dva režima: želatinska SDS-PAGE (zimogram) u jednoj dimenziji [124] i dvodimenzionalna želatinska PAGE [125].

Protokol:

Gel za razdvajanje se priprema kao i gel za razdvajanje u SDS-PAGE (odjeljak 4.1.2.1.1) sa tom razlikom što se umesto destilovane vode dodaje ista zapremina 0,3% rastvora želatina u vodi (finalna koncentracija želatina u gelu za razdvajanje iznosila je 0,1%). Gel za koncentrovanje priprema se na identičan način kao i gel za koncentrovanje u SDS-PAGE (odjeljak 4.1.2.1.1).

Uzorci se pripremaju mešanjem sa pet puta koncentrovanim redukujućim puferom za uzorke (rastvor H) i inkubiranjem na 60 °C 30 min.

Elektroforetsko razdvajanje proteina rađeno je na konstantnoj struji od 25 mA po gelu.

Postupak zimogramske detekcije proteinskih traka (negativno bojenje) prikazan je u tabeli 4.22.

Tabela 4.22. Faze detekcije proteinskih traka.

Faza	Rastvor	Vreme (min)
Ispiranje	Destilovana voda	3x5 min
Pufer optimalne pH vrednosti	0,1 M Tris pufer pH 8,0 ili 0,1 M natrijum-fosfatni pufer pH 7,2 sa 20 mM L-cisteinom	Preko noći
Bojenje	Rastvor I	15
Obezbojavanje	Rastvor J	30
Obezbojavanje	Rastvor K	Do obezbojenja

Prisustvo želatinolitičke aktivnosti identifikovano je kao prozirna traka na plavoj pozadini.

4.1.2.2.1. Denzitometrijska analiza zimogramskih gelova

Denzitometrijska analiza gelova urađena je korišćenjem programa Gel-Pro Analyzer 3.1 (Media Cybernetics, Silver Spring, MD, SAD) sa opcijom za analizu pojedinačnih traka.

4.1.2.3. Dvodimenzionalna zimogramska detekcija

Dvodimenzionalna elektroforeza (2D elektroforeza) podrazumeva izoelektrično fokusiranje (IEF) u prvoj i SDS-PAG elektroforezu u drugoj dimenziji, odnosno razdvajanje proteina na osnovu pI vrednosti u prvoj i na osnovu molekulske mase u drugoj dimenziji. U ovom radu rađena je modifikacija standardne 2D elektroforeze u kojoj je u gel za drugu dimenziju inkorporiran želatin (0,1%) u cilju zimogramske detekcije aktivnih želatinolitičkih proteaza.

4.1.2.3.1. Prva dimenzija - izoelektrično fokusiranje (IEF)

Priprema denaturišućeg urea-IEF gela prikazana je u tabeli 4.23. i tabeli 4.24. Korišćeni su amfoliti Ampholine® (GE Healthcare, Upsala, Švedska), pH opsega 3,5-10.

Tabela 4.23. Priprema gela za izoelektrično fokusiranje.

Komponente	7 mL gela	12 mL gela
Monomerni rastvor 30%	1,2 mL	2,0 mL
Urea	3,5 g	6,0 g
Amfoliti	0,35 mL	0,6 mL
Destilovana voda	2,85 mL	5,0 mL
TEMED	12 µL	20 µL
APS	45 µL	75 µL

Tabela 4.24. Priprema gela za koncentrovanje uzoraka (zapremine 3 mL).

Komponente	Zapremina
Monomerni rastvor 30%	400 µL
40 mM NaOH	1,35 mL
Destilovana voda	1,25 mL
TEMED	2 µL
APS	30 µL

U daljem radu korišćen je pufer za uzorke (tabela 4.25), kao i rastvori katolita i anolita. Rastvor katolita, gornjeg elektrolita (2x koncentrat 40 mM NaOH) razblažuje se neposredno pre upotrebe. Rastvor anolita, donjeg elektrolita, jeste 10 mM H₃PO₄.

Tabela 4.25. Rastvor A: pufer za pripremu uzoraka (PUZ) (2x koncentrat) (neredukujući – bez β-merkaptoetanol).

Urea	0,45 g
Amfoliti	5 µL
Triton X-100	20 µL
Bromfenol plavo i dH ₂ O	Do 1 mL

*Opciono se može dodati i 10 µL β-merkaptoetanol ukoliko su potrebni redukujući uslovi.

Postupak:

Prema podacima iz tabele, napravljeni su gelovi za IEF i naliveni između staklenih ploča sa spejserima debljine 1 mm. Ploče se nakon polimerizacije gela za koncentrovanje i nalivanja uzoraka u bunare urone u kadu sa donjim elektrolitom, dok se u gornji deo kade sipa gornji elektrolit. Korišćen je vertikalni Hoeffer Dual Gel Caster Mighty small SE 245 sistem po uputstvu proizvođača.

Uzorci se pripremaju mešanjem sa odgovarajućom zapreminom rastvora A. Nanošeno je po 30 µL uzoraka približne koncentracije proteina 0,1 do 1 mg/mL (određenoj Bredfordovom ili biuretskom metodom) (odeljak 4.1.1).

Pre nanošenja na gel, uzorci lateksa inkubirani su sa L-cisteinom (Sigma-Aldrich, Šajnhajm, Nemačka) u finalnoj koncentraciji od 52 mM.

Uslovi razdvajanja: 150 V, 50 mA, 100 W, 30 min - predfokusiranje amfolita i 200 V, 50 mA, 100 W, 150 min - razdvajanje proteina po pI vrednosti.

4.1.2.3.2. Određivanje pI vrednosti

Nakon IEF, gel se izdela na trake (bez uzorka i sa uzorcima). Trake sa uzorcima inkubiraju se u neredukujućem PUZ-u 30 min, a zatim u redukujućem PUZ-u (rastvor H) takođe uz inkubaciju od 30 min. Prazne trake gela iseku se na tračice dužine 1 cm i inkubiraju u 10 mM kalijum-hloridu. Nakon 30 min inkubacije meri se pH vrednost. Na osnovu izmerenih pH vrednosti formira se grafik zavisnosti pH vrednosti od udaljenosti

od anode. Iz jednačine prave računa se pI vrednost želatinolitičke proteaze tj. pI vrednost koja odgovara aktivnoj tački [125].

4.1.2.3.3. Druga dimenzija - želatinska SDS-PAGE

Trake sa uzorcima nakon inkubacije u puferu za uzorke postavljaju se na vrh gelova za razdvajanje i dalji tok elektroforeze je isti kao što je opisano za zimogramsku detekciju proteina u odeljku 4.1.2.2.

4.2. Izolovanje proteina

4.2.1. Izolovanje kolagenaze i ficina iz lateksa *Ficus carica*

4.2.1.1. Sakupljanje lateksa

Sveži lateks smokve (*F. carica*), koji se oslobađa nakon zasecanja ploda ili listova smokve, sakupljen je sa privatne plantaže u Baru, u Crnoj Gori. Uzorci lateksa sakupljeni su na svake dve nedelje u periodu od početka maja do polovine avgusta. Svaki put lateks je sakupljen sa tri ista drveta smokve, odnosno iz oko 10 plodova smokve. Za potrebe izolovanja kolagenolitičke proteaze i biohemijske karakterizacije, kao i za paralelno izolovanje ficina, zbog većeg prinosa lateksa korišćen je uzorak lateksa sakupljen sredinom avgusta. Gusta tečnost lateksa je sakupljana u mikrotubama od 1,5 mL i odmah nakon punjenja tuba, čuvan na -20 °C do trenutka korišćenja.

4.2.1.2. Priprema vodene frakcije lateksa

Zamrznuti lateks odleđen je na 4 °C i potom centrifugiran na 10 000 x g na 4 °C 60 min u cilju uklanjanja nerastvornih fragmenata ćelija (približno 20% mase). Odmašćivanje je rađeno sa dve zapremine petroletra da bi se izdvojio lipidni materijal i potpuno izbistrila vodena faza. Ovaj proces je ponovljen tri puta [126]. Alikvoti zapremine od po 1 mL vodene frakcije lateksa (u daljem tekstu samo lateks) čuvani su na -20 °C.

4.2.1.3. Frakcionisanje proteina lateksa gel-filtracijom

Nakon odleđivanja alikvoti lateksa preinkubirani su na sobnoj temperaturi sat vremena sa cisteinom u finalnoj koncentraciji od 52 mM da bi se aktivirao ficin, odnosno, da bi se redukovali dimeri ficina [127].

Potrebni rastvori i materijal:

0,1 M Tris pufer pH 8,0

Matriks Sephadex G-75

Kolona dimenzija 1 x 50 cm

Protokol:

Prethodno pripremljeni uzorak nanet je na kolonu za gel-filtraciju napakovanu Sephadex G-75 matriksom koji je ekvilibrisan sa 20 zapremina kolone (CV) 0,1 M Tris pufera pH 8,0. Frakcije koje su se sastojale od proteina sa kazeinolitičkom i želatinolitičkom aktivnošću spojene su i skoncentrovane ultrafiltracijom. Testovi za određivanje kazeinolitičke i želatinolitičke aktivnosti opisani su u odeljku 4.3.1.1. i 4.3.1.2.2. Sa tako pripremljenim uzorkom dalje je rađena kovalentna hromatografija (odeljak 4.2.1.4).

4.2.1.4. Prečišćavanje kolagenaze i ficina kovalentnom hromatografijom

Potrebni rastvori i materijal:

Pufer A: 0,1 M Tris pufer pH 8,0

Pufer B: 0,1 M Tris pufer pH 8,0 sa 20 mM L-cisteinom

Matriks tiol-Sepharose 4B

Kolona dimenzija 1 x 10 cm

Protokol:

Uzorak je nanet na kolonu napakovanu tiol-Sepharose matriksom koji je prethodno ekvilibrisan sa 20 CV pufera A. Nevezani proteini eluirani su sa matriksa puferom A, sve dok se vrednost apsorbancije na 280 nm nije spustila do manje od 0,05. Kovalentno vezana frakcija ficina eluirana je puferom B [72]. Frakcije su sakupljene pod protokom od 0,8 mL/min. Vezane frakcije (sa kazeinolitičkom aktivnošću, ficin) spojene su i analizirane elektroforetski. Želatinolitička aktivnost u frakcijama nevezanih proteina detektovana je zimografski (odeljak 4.1.2.2). Frakcije sa detektovanom

želatinolitičkom aktivnošću su spojene, skoncentrovane ultrafiltracijom (uz izmenu pufera u 50 mM natrijum-fosfatni pufera pH 7,0) i potom je sa njima rađena jonoizmenjivačka hromatografija (odjeljak 4.2.1.5).

4.2.1.5. Prečišćavanje kolagenaze jonoizmenjivačkom hromatografijom

Potrebni rastvori i materijal:

Pufer A: 50 mM natrijum-fosfatni pufer pH 7,0

Pufer B: 50 mM natrijum-fosfatni pufer pH 7,0 sa 0,5 M natrijum-hloridom

Matriks dietilaminoetil (DEAE)-Sephrose

Kolona dimenzija 1 x 10 cm

Protokol:

Uzorak je nanet na kolonu napakovanu DEAE-Sephrose matriksom koji je prethodno ekvilibrisan sa 10 CV pufera A. Nevezani proteini eluirani su sa matriksa puferom A, sve dok se vrednost apsorbancije na 280 nm nije spustila do manje od 0,05. Vezana kolagenaza eluirana je povećanjem jonske sile (puferom B). Frakcije su sakupljene pod protokom od 0,8 mL/min. Vezane frakcije (kolagenaza) spojene su i analizirane elektroforetski (odjeljak 4.1.2.1. i 4.1.2.2). Želatinolitička aktivnost u vezanim proteinima detektovana je zimogramski (odjeljak 4.1.2.2).

4.2.1.6. Gel-filtraciona analiza prečišćene kolagenaze

Analitička gel-filtracija prečišćene kolagenolitičke proteaze je rađena u 50 mM natrijum-fosfatnom puferu pH 7,0 na sistemu za tečnu hromatografiju visokih performansi (*engl.* High Performance Liquid Chromatography - HPLC) Akta Purifier 10 System (GE Healthcare, Upsala, Švedska) na koloni sa matriksom Superdex 75 PC 3.2/30 prema uputstvima proizvođača. Kalibracija kolone urađena je pomoću smeše standarda molekulske mase za gel-filtraciju (1 mg/mL svakog): goveđi serum albumin, karboanhidraza i citohrom c (Sigma–Aldrich, Štajnhajm, Nemačka).

4.2.2. Izolovanje papaina iz komercijalnog preparata

Papain je prečišćen iz komercijalnog preparata papaina (The British Drug House Ltd, London, Engleska) u cilju uklanjanja neaktivnog i denaturisanog papaina kao i obojenih jedinjenja male molekulske mase koja bi mogla da ometaju praćenje strukturnih promena papaina spektroskopskim metodama.

Protokol:

Papain je iz suvog komercijalnog praha ekstrahovan 30 min na 10 °C mešanjem na magnetnoj mešalici 100 mM Tris puferom pH 8,0 koji je sadržao 100 mM NaCl i 1 mM EDTA. Ekstrakt je centrifugiran 15 min na 4 000 x g na 10 °C i dobijeni bistri supernatant korišćen je u daljem radu. Papain je iz supernatanta istaložen dodavanjem 2 zapremine ledenog acetona i inkubiranjem 30 min na -20 °C. Istaloženi protein odvojen je centrifugiranjem 5 min na 10 000 x g na 4 °C, osušen i resuspendovan u 100 mM Tris puferu pH 8,0 koji je sadržao 100 mM NaCl i 1 mM EDTA. Urađena je kovalentna hromatografija korišćenjem matriksa tiol-Sepharose 4B (GE Healthcare, Upsala, Švedska). Matriks je ekvilibrisan sa 20 CV 100 mM Tris pufera pH 8,0 koji je sadržao 100 mM NaCl i 1 mM EDTA, nakon čega je nanet uzorak. Nevezani proteini eluirani su sa 20 CV startnog pufera, dok je kovalentno vezani papain eluiran startnim puferom sa 10 mM L-cisteinom. Homogenost prečišćenog proteina analizirana je metodom SDS-PAGE, na način koji je opisan u odeljku 4.1.2.1. Aktivnost je analizirana zimogramom (odeljak 4.1.2.2) i testom hidrolize sintetičkog supstrata BAPNA (4.3.1.2.1). Koncentracija prečišćenog proteina određena je Bredfordovom metodom, korišćenjem BSA kao standarda (odeljak 4.1.1.1). Papain je identifikovan peptidnim mapiranjem (odeljak 4.3.4).

4.3. Funkcionalni testovi

4.3.1. Testovi za određivanje enzimske aktivnosti

Enzimska aktivnost punog lateksa smokve određena je na uzorcima lateksa sakupljenim u različitim periodima (od formiranja do potpunog sazrevanja ploda) i to od 1. maja do 15. avgusta na svake dve nedelje. Upoređene su vremenske promene specifičnih aktivnosti lateksa.

4.3.1.1. Određivanje opšte proteolitičke aktivnosti

Opšta proteolitička aktivnost određena je korišćenjem kazeina (Sigma–Aldrich, Štajnhajm, Nemačka) kao supstrata na pH vrednosti 7,0 što je vrednost pH optimuma aktivnosti ficina [63]. Određivanje proteolitičke aktivnosti prema kazeinu kao supstratu određeno je sa uzorcima lateksa, ali i prečišćenog ficina. Svi uzorci lateksa razblaženi su u odnosu 1:200 u 0,1 M natrijum-fosfatnom puferu pH 7,0.

Protokol:

Pomešano je 50 μ L uzoraka sa 250 μ L rastvora supstrata (0,65% rastvor kazeina u 0,1 M natrijum-fosfatnom puferu pH 7,0 sa 10 mM L-cisteinom). Reakcione smeše inkubirane su 30 min na 37 °C. Reakcije su nakon toga stopirane dodavanjem 60 μ L 5% rastvora trihlorsirćetne kiseline (TCA). Smeše su centrifugirane 15 min na 12 000 x g. Izmerene su apsorbancije supernatanata na 280 nm. Za svaki uzorak pripremljene su i odgovarajuće slepe probe. Slepe probe enzima pripremljene su inkubiranjem 50 μ L odgovarajućih uzoraka 30 min na 37 °C bez supstrata. Nakon inkubacije najpre je dodato 60 μ L rastvora TCA, a potom i 250 μ L pufera. Slepe probe supstrata pripremljene su inkubiranjem rastvora kazeina sa puferom (bez enzima), nakon čega je dodat rastvor TCA. Apsorbancije obe slepe probe oduzimane su od apsorbancija uzoraka. Sve reakcione smeše i slepe probe rađene su u triplikatu.

Jedinica kazeinolitičke aktivnosti definisana je kao količina enzima koja daje apsorbanciju 1,0 na 280 nm za 1 min [63]. Specifična aktivnost izračunata je kao odnos enzimske aktivnosti i ukupnog sadržaja proteina u uzorku i izražena u U/mg.

4.3.1.2. Određivanje specifičnih proteolitičkih aktivnosti

Enzimska aktivnost lateksa i prečišćenih frakcija proteina određivana je, osim korišćenjem kazeina (nespecifični supstrat za proteaze), i prema supstratu BAPNA (Sigma–Aldrich, Štajnhajm, Nemačka).

Test hidrolize sintetičkog supstrata BAPNA korišćen je i za praćenje aktivnosti papaina, ficina i tripsina nakon svakog ciklusa izlaganja niskoj temperaturi.

4.3.1.2.1. Test hidrolize sintetičkog supstrata BAPNA

Test određivanja enzimske aktivnosti prema sintetičkom supstratu BAPNA rađen je prema protokolu koji su publikovali Devaraj i saradnici [63] uz manje modifikacije.

Protokol:

Supstrat BAPNA rastvoren je najpre u DMSO (5% od finalne zapremine), a potom razblažen 0,1 M Tris puferom pH 8,0 sa 10 mM L-cisteinom tako da je finalna koncentracija supstrata BAPNA iznosila 2 mM (sa 5% DMSO). Test je rađen inkubiranjem uzoraka sa 120 μ L sveže pripremljenog supstrata BAPNA u mikrotitar pločicama (Sarstedt, Njuton, Severna Karolina, SAD). Nakon 60 min inkubiranja na sobnoj temperaturi (22 °C), dodato je 100 μ L 50% sirćetne kiseline da bi se zaustavila reakcija. Izmerena je apsorbancija na 405 nm u čitaču za mikrotitar pločice LKB Micro plate reader 5060-006 (GDV, Rim, Italija). U test je uključena i odgovarajuća slepa proba.

Jedinica enzimske aktivnosti (U) definisana je kao količina enzima koja hidrolizuje 1 μ mol supstrata BAPNA po minutu (ekstinkcioni koeficijent *p*-nitroanilina: $\epsilon = 8800 \text{ cm}^{-1} \text{ M}^{-1}$ na 405 nm). Specifična aktivnost izračunata je kao odnos enzimske aktivnosti i ukupnog sadržaja proteina u uzorku i izražena kao U/mg.

4.3.1.2.2. Testovi hidrolize kolagena i želatina

Enzimska aktivnost prema kolagenu i želatinu određena je korišćenjem ninhidrinske metode prema protokolu koji su publikovali Zang i saradnici [128], uz male modifikacije.

Protokol:

Za potrebe određivanja kolagenolitičke aktivnosti, kolagen (tipa I iz repa pacova, Sigma–Aldrich, Štajnhajm, Nemačka) resuspendovan je u 50 mM Tris puferu pH 8,1 laganim mešanjem na 37 °C. Za potrebe određivanja želatinolitičke aktivnosti, želatin (iz goveđe kože, Merck, Darmstad, Nemačka) rastvoren je u istom puferu. Finalna koncentracija kolagena iznosila je 5 mg/mL, a želatina 20 mg/mL. Reakcione smeše sa po 100 μ L suspenzije kolagena, odnosno rastvora želatina, i po 10 μ L uzoraka inkubirane su 5 h na 37 °C uz blago mešanje. Potom je dodato 110 μ L 20% (m/v) PEG

6000 (Sigma–Aldrich, Štajnhajm, Nemačka) i smeše su inkubirane dodatnih sat vremena na 4 °C. Smeše su centrifugirane na 12 000 x g 30 min na 4 °C. U test su uključene i odgovarajuće slepe probe. Dvadeset mikrolitara supernatanta reakcionih smeša pomešano je sa 200 µL rastvora ninhidrinskog reagensa (koji se priprema mešanjem jednakih zapremina 4% rastvora ninhidrina u propilen-glikolu i 200 mM citratnog pufera pH 5,0 sa 0,16% SnCl₂), inkubirano na 100 °C 10 min i ohlađeno do sobne temperature. Smeše su potom razblažene sa po 400 µL 50% 1-propanola i izmerena je apsorbancija na 570 nm naspram slepe probe koja je sadržala samo ninhidrinski reagens, bez supstrata i uzorka.

Jedna arbitrarna jedinica aktivnosti digestije kolagena odnosno želatina (*engl.* Collagen Digestion Unit - CDU, odnosno Gelatin Digestion Unit - GDU), definisana je kao količina enzima koja oslobađa količinu peptida ekvivalentnu po boji ninhidrina kao i 1 µmol leucina za 5h. Specifična aktivnost izračunata je kao odnos enzimske aktivnosti i ukupnog sadržaja proteina u uzorku i izražena u U/mg.

4.3.1.2.3. Test hidrolize lanaca nativnog kolagena

Test hidrolize lanaca nativnog kolagena rađen je po protokolu koji su opisali Kim i saradnici uz manje modifikacije [105].

Protokol:

Reakcione smeše sastojale su se od 15 µL frakcije prečišćene kolagenaze koncentracije 0,1 mg/mL i 60 µL rastvora nativnog kolagena u 0,1 M Tris puferu pH 8,1 koncentracije 0,5 mg/mL. Nakon 16 h inkubiranja na 37 °C, reakcione smeše su pripremljene za SDS-PAGE analizu dodavanjem pufera za uzorke i inkubiranjem 5 min na 95 °C. Analizirane su i reakcione smeše u kojima je prečišćena frakcija kolagenaze preinkubirana u prisustvu inhibitora (odjeljak 4.3.2). Proizvodi digestije lanaca nativnog kolagena analizirani su na SDS-PAGE sa 8% gelom za razdvajanje (odjeljak 4.1.2.1).

U cilju ispitivanja sposobnosti kolagenaze da hidrolizuje nativni kolagen u regionu trostrukog heliksa, korišćena je i metoda FT-IR spektroskopije (odjeljak 4.5.3.1). Uzorci su pripremljeni na sledeći način:

- nativni kolagen (60 μ L koncentracije 5 mg/mL, resuspendovan u 0,1 M Tris puferu pH 8,1) nakon inkubiranja sa 15 μ L istog pufera u trajanju od 16h na 37 °C

- nativni kolagen (60 μ L koncentracije 5 mg/mL, resuspendovan u 0,1 M Tris puferu pH 8,1) nakon digestije sa kolagenazom (15 μ L koncentracije 0,1 mg/mL) u trajanju od 16h na 37 °C.

4.3.1.2.4. Test hidrolize κ -kazeina (koagulacija mleka)

Test hidrolize κ -kazeina rađen je po Beridžovom protokolu [129].

Protokol:

Beridžov test za određivanja koagulacione aktivnosti prema mleku (hidrolizom κ -kazeina), zasniva se na merenju vremena potrebnog za formiranje koaguluma (vreme koje protekne od trenutka mešanja uzorka lateksa sa mlekom do pojave pahuljičastog taloga kazeina). Obrano mleko (0,5 mL) sa 10 mM CaCl_2 kome je pH podešeno na 6,5 preinkubirano je na 37 °C. Dodato je 200 μ L uzorka lateksa sa 10 mM L-cisteinom. Reakciona smeša inkubirana je na 37 °C i mereno je vreme do pojave pahuljičastog taloga. Eksperiment je rađen u triplicatu. Postavljena je i pozitivna kontrola (mleko koagulisano himozinom koncentracije 0,044 mg/mL).

Jedinica koagulacione aktivnosti definisana je kao količina enzima potrebna da koaguliše 1 mL mleka u minutu na 37 °C. Specifična aktivnost izračunata je kao odnos enzimске aktivnosti i ukupnog sadržaja proteina i izražena je u U/mg.

4.3.2. Efekat inhibitora na enzimsku aktivnost

Testiran je efekat sledećih inhibitora na proteolitičku, želatinolitičku i kolagenolitičku aktivnost lateksa, kao i na želatinolitičku i kolagenotlitičku aktivnost prečišćene frakcije kolagenaze:

- 2 mM IAA (Sigma–Aldrich, Štajnhajm, Nemačka), inhibitor cistein-proteaza;
- 2 mM PMSF (Sigma–Aldrich, Štajnhajm, Nemačka), inhibitor serin-proteaza;
- 2 mM EDTA, inhibitor metaloproteaza;
- 22,5 μ M E-64 (Sigma–Aldrich, Štajnhajm, Nemačka), inhibitor cistein-proteaza;

- 14,6 μ M pepstatin A (Sigma–Aldrich, Štajnhajm, Nemačka), inhibitor aspartat-proteaza.

Protokol:

Alikvoti uzoraka razblaženih 0,1 M Tris puferom pH 8,0 inkubirani su u prisustvu inhibitora 30 min na sobnoj temperaturi (22 °C) pre testova enzimske aktivnosti. Preostala aktivnost izračunata je kao procenat enzimske aktivnosti u odnosu na kontrolni uzorak u koji nije dodat inhibitor (100% aktivnost).

Efekat inhibitora na prečišćenu frakciju kolagenaze određen je zimografski (odeljak 4.1.2.2). Nakon elektroforeze i ispiranja gelova, isti su isečeni na delove sa triplikatima uzoraka i svaki deo gela zasebno je inkubiran 16h u puferu u koji je dodat odgovarajući inhibitor u navedenoj koncentraciji. Nakon inkubiranja, gelovi su bojeni rastvorom boje CBB. Inhibicija je izražena kao procenat intenziteta proteolitičkih traka (određen denzitometrijski - odeljak 4.1.2.2.1) u prisustvu inhibitora u poređenju sa kontrolom (bez inhibitora).

Poređenje uticaja četiri različita inhibitora serin-proteaza (2 mM PMSF, 0,1 mM Pefabloc SC, 2 mM benzamidin-hidrohlorid i 1 mM inhibitor tripsina iz soje (*engl.* soybean trypsin inhibitor- STI)) urađeno je analizom proizvoda hidrolize kolagena na SDS-PAGE sa 8% gelom za razdvajanje (odeljak 4.1.2.1).

4.3.3. Određivanje pH i temperaturnog optimuma i stabilnosti

Karakterisanje kolagenaze urađeno je i u kontekstu određivanja pH i temperaturnog optimuma enzimske aktivnosti, kao i stabilnosti.

U cilju određivanja optimalne pH vrednosti za aktivnost enzima, pH vrednost reakcione smeše sa 2% želatinom varirana je u opsegu od 3,0 do 9,5, dok je za ispitivanje pH stabilnosti enzim preinkubiran na određenoj pH vrednosti (od 3,0 do 9,5) 30 min, nakon čega je enzimska aktivnost merena na pH 8,0 (odeljak 4.3.1.2.2). Korišćeni su sledeći puferi: 50 mM natrijum-citratni pufer (pH 3,0 do 6,0), 50 mM natrijum-fosfatni pufer (pH 6,5 do 7,0), 50 mM Tris pufer (pH 8,0 do 9,0) i 50 mM glicinski pufer (pH 9,5).

U cilju određivanja optimalne temperature za aktivnost enzima, reakcione smeše inkubirane su na temperaturama od 20 do 80 °C, dok je za određivanje termostabilnosti enzim preinkubiran 30 min na određenoj temperaturi od 20 do 90 °C, nakon čega je enzimsko aktivnost merena na 37 °C.

Preostala aktivnost izračunata je kao odnos enzimsko aktivnosti izmerene nakon svakog inkubiranja i vrednosti enzimsko aktivnosti pre inkubiranja na određenoj vrednosti pH/temperature. Vrednosti su izražene kao procenti maksimalne aktivnosti (početne aktivnosti).

4.3.4. Tehnika identifikovanja peptida (*engl.* Trypsin Mass Fingerprinting- TMF)

Tehnika otiska prsta za identifikovanje peptida rađena je pri identifikaciji papaina nakon prečišćavanja, kao i u cilju provere aktivnosti tripsina nakon izlaganja niskim temperaturama. Kao supstart za digestiju tripsinom korišćen je BSA.

4.3.4.1. Digestija proteina

Digestija proteina rađena je po instrukcijama proizvođača tripsina za sekvenciranje (Sigma-Aldrich, Štajnhajm, Nemačka). Naime, 1 mg proteina inkubiran je sa 100 µL 8 M uree i redukovan ditiotreitolom. Rastvor proteina je potom razblažen 50 mM amonijum-bikarbonatom (AB) 10 puta. U ovako pripremljene rastvore proteina dodat je, u slučaju identifikacije papaina, tripsin za sekvenciranje resuspendovan u 1 mM HCl po uputstvu proizvođača, dok je u slučaju praćenja aktivnosti tripsina nakon izlaganja niskoj temperaturi dodat svaki od različito tretiranih rastvora tripsina za sekvenciranje:

- netretirani tripsin u 1 mM HCl;
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB (kontrola);
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB sa 0,3 M glicerolom (AB-G);
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB sa 0,1 M lizinom (AB-K);

i to u odnosu protein:tripsin 150:1. Smeše su digestovane 12h na 37 °C.

4.3.4.2. Tečna hromatografija-masena spektrometrija (LC-MS)

Peptidi dobijeni digestijom proteina injektovani su na reverzno-faznu C18 kolonu (RR HT, 1,8 μm , 4,6 x 50 mm) kuplovanu sa Zorbax Eclipse XDB-C18 koji je instaliran sistem 1200 series HPLC (Agilent Technologies, Santa Klara, Kalifornija, SAD). Uzorci su razdvojeni gradijentom acetonitrila (5-95% 10 min sa 0,2% mravljom kiselinom i dodatnih 5 min sa 95% acetonitrilom). Maseni spektrometar (6210 Time-of-Flight (TOF) LC-MS system (G1969A; Agilent Technologies, Santa Clara, CA, USA)) podešen je na režim pozitivne elektron-sprej jonizacije (ESI) sa naponom na vrhu kapilare od 4000 V, naponom fragmentora od 200 V i opsegom m/z od 100 do 3200. Dobijene mase peptida pretražene su u SwissProt bazi proteinskih sekvenci korišćenjem programa MASCOT.

4.4.Optimizovanje metodologije za ispitivanje stabilnosti serin- i cistein-proteaza

4.4.1. Ispitivanje kinetičke stabilnosti papaina i ficina

4.4.1.1. Termalna inaktivacija papaina i ficina

Za konstruisanje krivih termalne inaktivacije papaina i ficina, pripremljeni su rastvori svakog od enzima u koncentraciji 0,5 mg/mL u 0,1 M Tris puferu pH 8,0 koji je sadržao 0,1 M NaCl, 1 mM EDTA i 10 mM L-cistein.

Protokol:

Pripremljeni rastvori papaina i ficina inkubirani su 30 min na odgovarajućim temperaturama u intervalu 15-100 °C. Nakon inkubacije na odgovarajućoj temperaturi, rastvor je najpre ohlađen do sobne temperature na kojoj je izmerena aktivnost korišćenjem supstrata BAPNA kao što je opisano u odeljku 4.3.1.2.1. Merenja su izvedena u triplikatu. Izračunat je procenat preostale aktivnosti u odnosu na uzorke inkubirane na 40 °C (maksimalna aktivnost).

4.4.1.2. Određivanje konstanti brzina inaktivacije papaina i ficina

Konstante brzina inaktivacije papaina i ficina određene su na četiri različite temperature sa opadajućeg dela krive inaktivacije (75, 80, 85 i 90 °C za papain, i 60, 65, 70 i 75 °C

za ficin). Rastvori papaina i ficina pripremljeni su na prethodno opisan način (odjeljak 4.4.1).

Protokol:

Rastvori papaina i ficina inkubirani su na odgovarajućim temperaturama i nakon određenih vremenskih intervala (1 minut do 45 min), uzimani su alikvoti. Nakon hlađenja do sobne temperature, u alikvotima je merena aktivnost korišćenjem supstrata BAPNA (odjeljak 4.3.1.2.1). Procenat preostale aktivnosti izračunat je u poređenju sa aktivnošću uzorka pre inkubacije na određenoj temperaturi. U cilju izračunavanja konstanti brzina inaktivacije konstruisan je grafik zavisnosti logaritma preostale aktivnosti od vremena inkubacije (izraženog u minutima). Vrednosti konstanti brzina izračunate su iz nagiba dobijenih linearnom regresionom analizom:

$$\log (100 A/A_{\max}) = - (k/2,303) t$$

gde A predstavlja apsorbanciju na 405 nm izmerenu nakon određenog perioda inkubacije, A_{\max} predstavlja maksimalnu apsorbanciju koja je dobijena pre inkubacije, k predstavlja konstantu brzine inaktivacije, dok t predstavlja vreme inkubacije za termalnu inaktivaciju [130].

4.4.1.3. Konstruisanje Arenijusovog grafika za inaktivaciju papaina i ficina

Aktivaciona energija za inaktivaciju papaina i ficina izračunata je konstruisanjem Arenijusovog grafika koji podrazumeva grafik zavisnosti prirodnog logaritma konstanti brzina inaktivacije ($\ln k$) od recipročne vrednosti apsolutne temperature (T^{-1}). Aktivacione energije izračunate su iz nagiba Arenijusovog grafika u skladu sa jednačinom:

$$\ln k = - E_a/RT + c$$

gde R predstavlja univerzalnu gasnu konstantu (8,314 J/molK) i T predstavlja apsolutnu temperaturu u kelvinima (K). Nagibi i odgovarajuće standardne greške dobijeni su iz linearne regresione analize [130].

4.4.1.4. Ispitivanje rezistencije na denaturaciju detergentom (SDS)

Rezistencija papaina na SDS ispitana je analizom uticaja temperature na vezivanje SDS-a i to na osnovu različite mobilnosti papaina u SDS-PAGE (odjeljak 4.1.2.1), ali i na osnovu praćenja sklonosti ka zauzimanju različitih sekundarnih struktura metodom FT-IR spektroskopije.

4.4.1.4.1. Infracrvena spektroskopija sa Furijeovom transformacijom (FT-IR)

Infracrvena spektroskopija sa Furijeovom transformacijom sa umanjenom ukupnom refleksijom (*engl.* attenuated total reflectance - ATR) i rezolucijom 2 cm^{-1} rađena je na instrumentu Nicolet 6700 FT-IR sa softverom OMNICS verzija 7.0 (Thermo Scientific, SAD). Koncentracija proteina u uzorcima iznosila je oko 1 mg/mL . Na postolje za snimanje spektara sa dijamantskim kristalom (Smart Orbit, Thermo Scientific, USA) nanošeno je $5\text{--}10\text{ }\mu\text{L}$ rastvora proteaza ili pufera u kojima su rastvorene. Nakon nanošenja rastvora proteina rastvarač (pufer) uparavan je strujom azota sve do dobijanja tankog ATR filma. Za svaki spektar sakupljeno je po 64 snimka. Spektri odgovarajućih pufera oduzimani su od spektara proteina jer apsorbancija vode interferira sa apsorbancijom proteina u amidnom I regionu. Nakon oduzimanja vode (kriterijum za adekvatno oduzimanje vode je uklanjanje trake oko 2200 cm^{-1} , kao i ravna bazna linija u regionu između 1800 cm^{-1} i 2000 cm^{-1}) spektri su obrađeni u softveru OMNICS da bi se uklonio šum (za faktor $12,535\text{ cm}^{-1}$). Takođe je urađena i automatska korekcija za dijamantski kristal.

4.4.1.4.2. Analiza tendencije za zauzimanje određenih sekundarnih struktura

Snimljeni su FT-IR spektri sledećih uzoraka papaina:

- papain inkubiran 30 min na 25, 60, odnosno $90\text{ }^{\circ}\text{C}$;
- papain inkubiran 30 min na 25, 60, odnosno $90\text{ }^{\circ}\text{C}$ u prisustvu 1% SDS.

Na dijamantski kristal FT-IR spektrofotometra naneto je po $15\text{ }\mu\text{g}$ uzoraka.

Dobijeni originalni FT-IR spektri su nakon obrade opisane u odeljku 4.4.1.4.1. i oduzimanja pufera (vode) obrađeni metodom Furijeove samodekonvolucije korišćenjem

programa OMNICS sa sledećim parametrima: širina traka na polovini visine podešena je na 13 cm^{-1} , a faktor povećanja rezolucije na 2,4, kao što je prethodno opisano [131].

Da bi se uporedile promene sekundarnih struktura u uzorcima papaina inkubiranim 30 min na 25, 60 i 90 °C sa ili bez prisustva 1% SDS-a, izračunat je odnos intenziteta između traka amidnog I regiona, specifičnih za svaku od sekundarnih struktura, i trake koja predstavlja maksimum amidnog II regiona (1520 cm^{-1}) koja se koristi kao interni standard [132, 133]; ovaj odnos daje informacije o tendenciji zauzimanja određene sekundarne strukture.

4.4.2. Tehnike za praćenje termalne denaturacije papaina

4.4.2.1. Diferencijalna UV spektroskopija

Merenje promena UV apsorpcije papaina rađeno je na spektrofotometru Evolution 300 (Thermo Fisher Scientific, Medison, SAD) korišćenjem kvarcne kivete širine 1 cm. Spektrofotometar je opremljen uređajem za kontrolu temperature koji je omogućavao programiranje temperaturnog režima za nekoliko kiveta istovremeno. Merenje apsorpcije je, stoga, bilo moguće izvesti u funkciji od temperature.

Protokol:

Najpre su snimljeni spektri nativnog papaina (sobna temperatura) i termalno denaturisanog papaina (99 °C) i to u opsegu talasnih dužina od 230 do 300 nm u Tris puferu pH 8,0. Koncentracija papaina iznosila je 1 mg/mL. Spektar samog pufera služio je kao slepa proba. Termalna denaturacija papaina je nakon toga praćena merenjem apsorpcije na 230, 267 i 286 nm u funkciji od temperature (5-99 °C). Diferencijalni UV spektar izračunat je i konstruisan oduzimanjem apsorpcija potpuno nativnog i potpuno denaturisanog papaina na svim talasnim dužinama na kojima je spektar sniman [4]. Talasna dužina od 267 nm izabrana je za dalju analizu jer je na ovoj talasnoj dužini dobijena najveća razlika u ekstinkcionim koeficijentima nativnog i termalno denaturisanog papaina. Brzina povećanja temperature iznosila je 0,5 °C/min.

Na osnovu UV spektroskopije izračunata je Gibsova slobodna energija (ΔG°) denaturacije papaina na različitim temperaturama. Najpre je izračunata frakcija termalno denaturisanog papaina (F_d) i to iz jednačine:

$$F_d = (Y_N - Y_{obs}) \times (Y_N - Y_D)^{-1}.$$

U datoj jednačini Y_{obs} predstavlja očitanu vrednost apsorbancije, dok Y_N i Y_D predstavljaju vrednosti apsorbancije karakteristične za potpuno nativan, odnosno potpuno denaturisani papain. Potom je izračunata ravnotežna konstanta procesa denaturacije papaina na osnovu jednačine:

$$K = F_d (1 - F_d)^{-1}.$$

Iz dobijenih vrednosti F_d i K izračunata je i Gibsova slobodna energija denaturacije papaina na osnovu jednačine:

$$\Delta G^\circ = -RT \ln K$$

gde R predstavlja univerzalnu gasnu konstantu (8,314 J/molK), a T predstavlja apsolutnu temperaturu u K [134].

Na osnovu izračunatih frakcija denaturisanog proteina, konstruisana je kriva termalne denaturacije (zavisnost procenta native strukture od temperature) u opsegu 5-99 °C.

4.4.2.2. Diferencijalna skenirajuća kalorimetrija (engl. Differential Scanning Calorimetry - DSC)

Termogrami papaina dobijeni su DSC metodom na instrumentu DSC Q1000 serije (TA Instruments, Njukasl, Delaver, SAD) sa uređajem za automatsko uzimanje uzoraka i sistemom za hlađenje (RSC, TA Instruments, Njukasl, Delaver, SAD).

Protokol:

Odmereno je približno 7 mg rastvora papaina koncentracije 10 mg/mL u 50 mM Tris puferu pH 8,0 na aluminijumsku kivetu i hermetički zatvoreno aluminijumskim poklopcem. Referentna kiveta ispunjena je istom zapreminom pufera u kome je papain rastvoren (slepa proba). Uzorak i slepa proba temperirani su najpre na 10 °C, a potom zagrevani do 90 °C brzinom od 1 °C/min. Temperaturni maksimum endotermalnog prelaza koji je karakterističan za termalnu denaturaciju papaina izračunat je upotrebom softvera TA Instruments Universal Analysis 2000 (verzija 4.1 D).

4.5. Tehnike za ispitivanje stabilnosti serin- i cistein-proteaza na niskim temperaturama

4.5.1. Praćenje aktivnosti proteaza na niskim temperaturama

Rastvori prečišćenih proteaza (ficina, papaina i kolagenaze) napravljeni su u 0,1 M Tris puferu pH 8,0, što je pH vrednost bliska optimalnoj vrednosti za sve navedene proteaze. U slučaju tripsina, napravljeni su rastvori niske pH vrednosti (u 1 mM HCl) i blago alkalne pH vrednosti (u 100 mM amonijum-bikarbonatnom puferu pH 8,0) sa i bez krioprotektanata (tabela 4.26).

Tabela 4.26. Krioprotektanti korišćeni za stabilizaciju tripsina.

Aditivi – polioli i polimeri	Finalna koncentracija u 100 mM AB
Propilen-glikol (PG)	0.3 M
Glicerol (G)	0.3 M
Polietilen-glikol 6 000 (PEG)	0.002 M
Aditivi - aminokiseline	
Lizin (K)	0.1 M
Arginin (R)	0.1 M

Ovako pripremljeni uzorci zamrzavani su brzinom od 1 °C/min, i odmrzavani brzinom od > 10 °C/min [46]. Postupak zamrzavanja/odmrzavanja ponovljen je nekoliko puta, a nakon svakog odmrzavanja, kao i pre prvog zamrzavanja, određena je aktivnost proteaza specifičnim testom. Uzorci su analizirani odmah po odmrzavanju, da bi se ograničilo inkubiranje tripsina na sobnoj temperaturi na maksimalno 2 minuta. Aktivnost ficina, papaina i tripsina određena je testom hidrolize supstrata BAPNA (odeljak 4.3.1.2.1), dok je aktivnost kolagenaze praćena zimografski (odeljak 4.1.2.2). Početna aktivnost posmatrana je kao 100% aktivnosti, dok su aktivnosti izmerene nakon svakog ciklusa zamrzavanja/odmrzavanja izražavane kao procenti preostale aktivnosti (u odnosu na početnu aktivnost). Svi eksperimenti rađeni su u triplikatu.

4.5.2. Praćenje autoproteolize proteaza na niskim temperaturama

U slučaju tripsina, praćenje autoproteolize rađeno je na sledećim uzorcima:

- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB (kontrola);

- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB sa 0,3 M glicerolom;
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB sa 0,1 M lizinom.

4.5.2.1. Vezivanje boje

Za ispitivanje autoproteolize uzimani su alikvoti rastvora proteaza pre zamrzavanja i nakon svakog ciklusa zamrzavanja/odmrzavanja. Odmah nakon odmrzavanja, po 10 μ L svakog rastvora proteina (triplikati) koncentracije 0,2 mg/mL pomešano je sa po 200 μ L rastvora CBB (0,125% CBB R-250 u 24% rastvoru etanola i 48% rastvoru fosforne kiseline) u bunarima mikrotitar pločice. Nakon 5 min inkubiranja sa bojom, merena je apsorbanacija na 620 nm. Rezultati merenja apsorbanacije izraženi su kao relativna apsorbanacija u procentima (u odnosu na izmerenu početnu vrednost apsorbanacije).

4.5.2.2. Reverzno-fazna tečna hromatografija (engl. Reverse-phase Liquid Chromatography - RP-HPLC)

Reverzno-fazna hromatografija nezamrzanih (nativnih) proteaza i zamrzanih proteaza (nakon uzastopnih ciklusa zamrzavanja/odmrzavanja) koncentracije oko 1 mg/mL rađena je na sistemu Äkta Purifier 10 (GE Healthcare, Upsala, Švedska) korišćenjem kolone Discovery® BIO Wide Pore C5-5 10 cm \times 4,6 mm, 5 μ m (Supelco, Bellefonte, Pensilvanija, SAD). Proteini su eluirani gradijentom acetonitrila (0-90% dužine 10 CV u prisustvu 0,1% trifluorosirćetne kiseline).

Reverzno-fazna hromatografija rađena je i u cilju karakterisanja izolovanog ficina, odnosno ispitivanja prisustva različitih izoformi, kao i detektovanja produkata autolize u slučaju papaina i ficina izlaganih niskim temperaturama.

4.5.2.3. Gel-filtracija

U cilju ispitivanja autoproteolize, odnosno očuvanosti primarne strukture u slučaju tripsina, korišćena je i gel-filtracija visokih performansi, metoda koja je u ovoj studiji primarno korišćena za ispitivanje agregiranja model proteaza.

Uzorci:

- netretirani tripsin u 1 mM HCl;
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 1 mM HCl;

- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB;
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB sa 0,3 M glicerolom;
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB sa 0,1 M lizinom;

Da bi se prevenirala dalja autoproteoliza tokom same hromatografske procedure, svi uzorci su nakon poslednjeg odmrzavanja tretirani sa 1 mM PMSF pre nanošenja na kolonu.

Gel-filtraciona hromatografija navedenih uzoraka rađena je na sistemu Äkta Purifier 10 (GE Healthcare, Upsala, Švedska) korišćenjem kolone Superdex 75 PC 3.2/30. Kolona je ekvilibrisana 0,1 M Tris puferom pH 8,0. Na kolonu je naneto po 10 µL rastvora proteaza koncentracije 1 mg/mL pri protoku od 0,05 mL/min. Kolona je kalibrisana markerima molekulske mase (svaki u koncentraciji od 1 mg/mL): BSA (67 kDa), karbonska anhidraza (29 kDa), citohrom c (14 kDa) i aprotinin (6,5 kDa) (Sigma–Aldrich, Štajnhajm, Nemačka). Za određivanje mrtve zapremine kolone korišćen je plavi dekstran (Sigma–Aldrich, Štajnhajm, Nemačka). Elucione zapremine standarda molekulskih masa iskorišćene su za kreiranje kalibracione prave ($V_e/V_t = f(\log M_w)$). Sa kalibracione prave određena je molekulska masa proteaza.

4.5.2.4. SDS-PAGE

U slučaju tripsina, za ispitivanje autoproteolize, odnosno očuvanosti primarne strukture korišćena je i SDS-PAGE (odjeljak 4.1.2.1) istih uzoraka tripsina kao što je opisano u odeljku 4.2.3.

4.5.3. Praćenje promena sekundarnih struktura proteaza - Infracrvena spektroskopija sa Furijeovom transformacijom (FT-IR)

4.5.3.1. Snimanje spektara

Uzorci:

A) Papain:

- netretirani papain (0,7 mg/mL) u 100 mM Tris puferu pH 8,0;

- papain (0,7 mg/mL) nakon 6 ciklusa zamrzavanja/odmrzavanja u 100 mM Tris puferu pH 8,0;

B) Ficin:

- netretirani ficin (0,7 mg/mL) u 100 mM Tris puferu pH 8,0;
- ficin (0,7 mg/mL) nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM Tris puferu pH 8,0;

C) Tripsin:

- netretirani tripsin;
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 1 mM HCl;
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB sa 0,3 M glicerolom;
- tripsin nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM AB sa 0,1 M lizinom;

D) Kolagenaza:

- netretirana kolagenaza (0,1 mg/mL) u 100 mM Tris puferu pH 8,0;
- kolagenaza (0,1 mg/mL) nakon 7 ciklusa zamrzavanja/odmrzavanja u 100 mM Tris puferu pH 8,0.

Snimanje FT-IR spektara, kao i obrada (uklanjanje šuma, ATR korekcija za dijamantski kristal i oduzimanje pufera (vode)) za sve navedene uzorke urađena je kao što je opisano u odeljku 4.4.1.4.1. Za uzorak tripsina sa lizinom bila je potrebna dodatna korekcija za apsorpciju lizina u amidnom I regionu (između 1700 cm^{-1} i 1600 cm^{-1}).

4.5.3.2. Analiza sekundarnih struktura

Obrada i analiza snimljenih FT-IR spektara rađena je u programu OMNICS kao što je opisano u odeljku 4.4.1.4.1. Region između 1700 cm^{-1} i 1600 cm^{-1} (amidni I region) razložen je na komponente od kojih je sastavljen originalni spektar, a potom su komponente (Lorencijeve krive) fitovane tako da se dobije visoka podudarnost sa originalnim spektrom uz minimalnu grešku. Nakon adekvatnog fitovanja ove krive su dodeljene odgovarajućim sekundarnim strukturama. Procenat površine koju zahvataju

pojedinačne Lorencijeve krive u odnosu na ukupnu površinu ispod originalnog spektra, predstavlja i procenat zastupljenosti pojedinačne sekundarne strukture. Na taj način omogućena je kvantitativna analiza sadržaja sekundarnih struktura. Raščlanjavanju originalnih spektara na pikove/komponente karakterističnih položaja i širina, prethodila je matematička operacija konvertovanja spektra u drugi izvod spektra (Savitsky-Golay derivativni spektar, trećeg polinomskog reda u sedam tačaka). Dodeljivanje dobijenih pikova određenim sekundarnim strukturama urađeno je u skladu sa prethodno publikovanim literaturom [56, 135-137].

4.5.3.3. Korelacija spektara

U cilju računanja stepena strukturne sličnosti između različito tretiranih uzoraka tripsina, upoređivani su drugi izvodi spektara kao što je opisano u prethodno publikovanoj literaturi [138, 139]. Koeficijenti korelacije spektara izračunati su na osnovu jednačine:

$$r = \frac{\sum^n x_i y_i}{\sqrt{\sum x_i^2 y_i^2}}$$

gde x_i i y_i predstavljaju vrednosti apsorbancija spektara koji se porede na i -toj poziciji frekvencije. Izračunavanje koeficijenata urađeno je u okviru amidnog I regiona.

4.5.4. Praćenje agregiranja proteaza usled izlaganja niskim temperaturama

Ispitivanje formiranja agregata proteaza nakon izlaganja niskoj temperaturi, rađeno je gel-filtracijom visokih performansi na način koji je opisan u odeljku 4.5.2.3. Hromatografija je urađena sa uzorcima papaina i ficina pre i nakon izlaganja uzastopnim ciklusima zamrzavanja/odmrzavanja. Određivanjem površina ispod pikova, određen je procenat agregiranja (koji zauzima pik eluiran u mrtvoj zapremini kolone).

4.5.5. Određivanje aktivacione energije inaktivacije tripsina niskom temperaturom

Aktivacione energije inaktivacije različito tretiranih uzoraka tripsina izračunate su na osnovu metode Borčarda i Danijelsa [140].

Navedeni pristup Borčarda i Danijelsa polazi od pretpostavke da reakcija prati kinetiku n -tog reda, te da sledi navedenu jednačinu:

$$\frac{d\alpha}{dt} = k(T)(1 - \alpha)^n \quad (a)$$

gde $d\alpha/dt$ predstavlja brzinu reakcije, α stepen konverzije, $k(T)$ specifičnu konstantu brzine na temperaturi T , dok n predstavlja red reakcije [140]. Pretpostavljajući da reakcija prati Arenijusovu zavisnost [141]:

$$k(T) = Ze^{-\frac{Ea}{RT}} \quad (b)$$

logaritam od jednačine (a) daje:

$$\ln\left(\frac{d\alpha}{dt}\right) = \ln(Z) - \frac{Ea}{RT} + n\ln(1 - \alpha)$$

$$\ln(k(T)) = \ln\left(\frac{d\alpha}{dt}\right) - n\ln(1 - \alpha)$$

čijim se kombinovanjem dobija:

$$\ln(k(T)) = \ln(Z) - \frac{Ea}{RT}$$

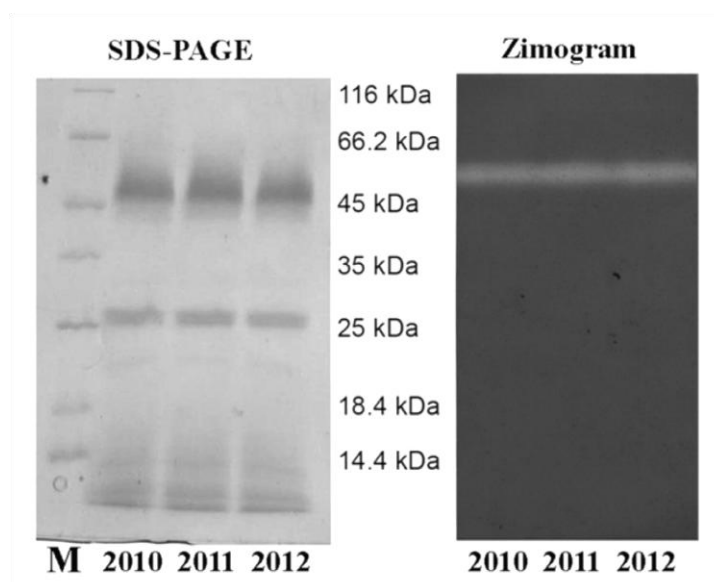
Zavisnost $\ln(k(T))$ od $1/T$ za vrednosti α između 0,1 i 0,5 za vrednost n , jeste linearna i daje kao nagib Ea/R , odakle se dalje može izračunati aktivaciona energija [140].

5. REZULTATI

5.1. Određivanje proteolitičke aktivnosti lateksa smokve

Jedan od polaznih materijala ovog istraživanja bio je lateks smokve (*Ficus carica*), bogat izvor proteina, pre svega proteolitičkih enzima. U cilju detektovanja, a potom i karakterisanja različitih proteolitičkih aktivnosti ekstrakta lateksa smokve, ispitana je sposobnost hidrolize nekoliko proteinskih supstrata.

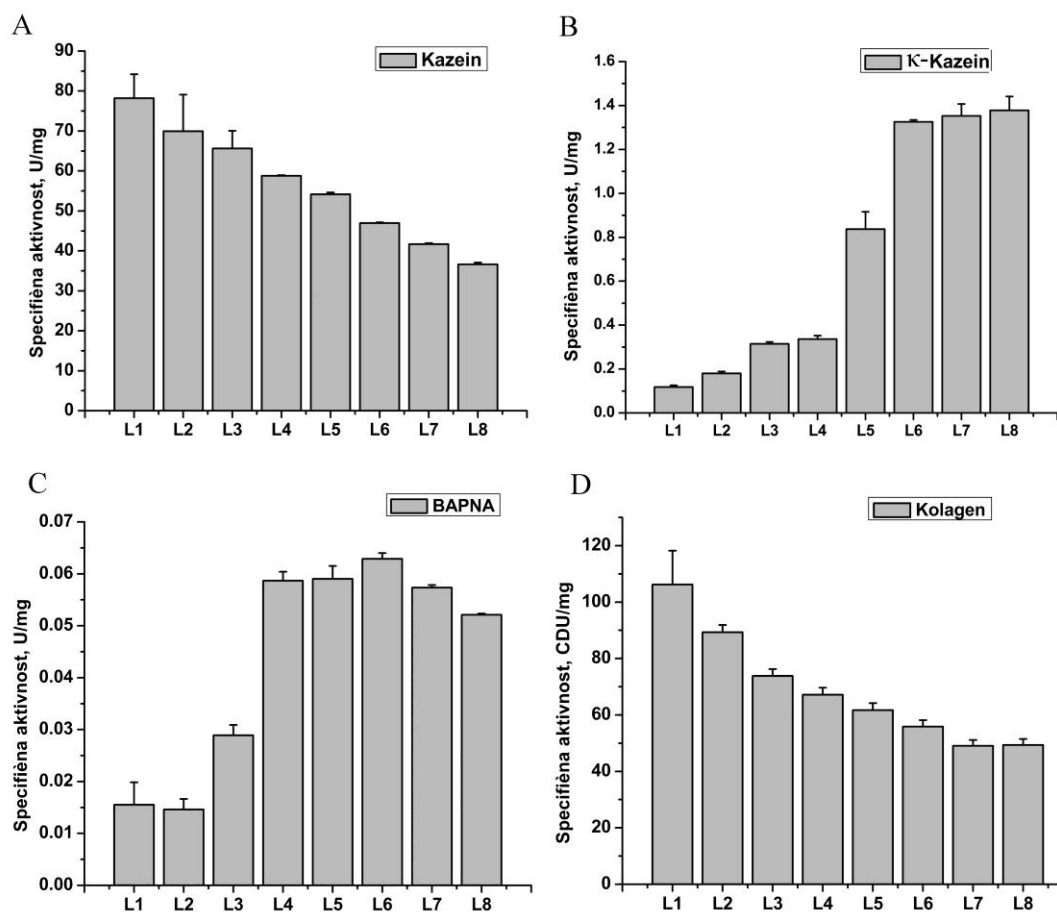
Ekstrakt lateksa smokve okarakterisan je u kontekstu detektovanja i kvantifikovanja aktivnosti prema različitim proteaznim supstratima. Proteinski profil i rezultati želatinolitičke aktivnosti punog lateksa sakupljenog u istom periodu tri različite godine prikazani su na slici 5.1.



Slika 5.1. A) SDS-PAGE i B) zimogram (sa želatinom inkorporiranim u gel za elektroforezu) proteina lateksa smokve. M - standardi molekulske mase. Prikazani su uzorci lateksa sakupljeni u tri uzastopne godine (2010, 2011. i 2012).

SDS-PAGE celokupnog ekstrakta lateksa (bez prečišćavanja na pojedinačne komponente) otkriva prisustvo glavnih proteinskih traka na pozicijama koje odgovaraju masama od oko 48 kDa i 25 kDa, kao i barem tri proteinske/peptidne trake molekulske mase ispod 14,4 kDa (slika 5.1). Zimogramska detekcija pokazuje da je hidrolitička aktivnost prema želatinu prisutna, i to na poziciji koja odgovara masi od oko 48 kDa (slika 5.1).

Kako se kazeinolitička aktivnost lateksa smokve tradicionalno pripisuje ficinu [85], nakon detektovanja hidrolitičke aktivnosti lateksa smokve prema želatinu kao supstratu, sledeći cilj je bilo ispitivanje potencijalne aktivnosti ekstrakta lateksa smokve u hidrolizi nativnog kolagena. Spektar različitih aktivnosti koje lateks ispoljava i intenzitet istih prikazan je u zavisnosti od perioda sakupljanja lateksa na slici 5.2.



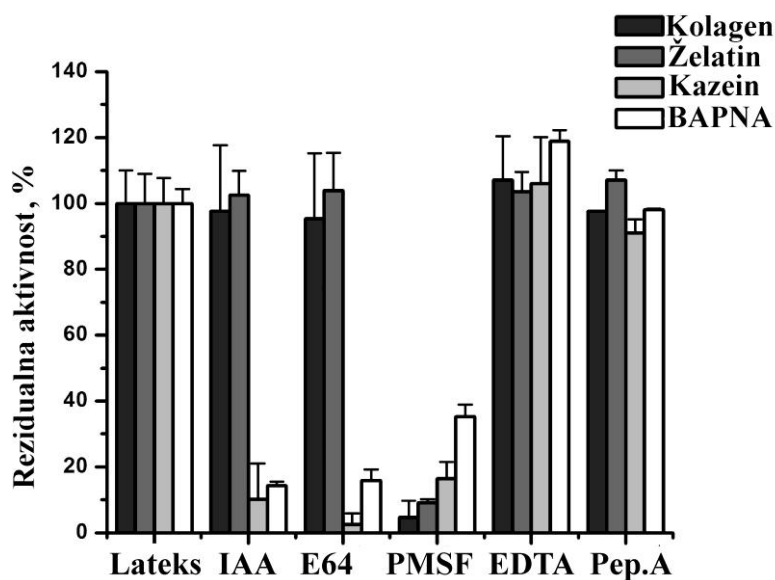
Slika 5.2. Specifična aktivnost lateksa smokve prema različitim supstratima: A) kazeinu, B) κ -kazeinu mleka, C) sintetičkom supstratu BAPNA i D) kolagenu. Specifična aktivnost izražena je kao ukupna aktivnost po masi (mg) ukupnih proteina. Definisanje različitih jedinica dato je u eksperimentalnom delu. L1-L8: uzorci lateksa sakupljeni na svake dve nedelje u periodu od 1. maja do 15. avgusta. Prikazani rezultati predstavljaju srednje vrednosti \pm standardne devijacije rezultata dobijenih iz tri nezavisna eksperimenta.

Histogramski prikaz različitih proteolitičkih aktivnosti lateksa smokve pokazuje da lateks smokve ima sposobnost hidrolize svih navedenih supstrata. Uočavaju se razlike u specifičnim aktivnostima u zavisnosti od perioda sakupljanja lateksa. Trend porasta

aktivnosti uočava se kod koagulacione aktivnosti mleka (slika 5.2B). S druge strane, hidroliza supstrata BAPNA mnogo je izraženija u uzorcima sakupljenim od 15. juna do 15. avgusta u odnosu na prolećne uzorke (slika 5.2C). Specifična aktivnost lateksa prema kazeinu prati pravilan trend opadanja bivajući najviša na samom početku razvoja ploda (oko 80 U/mg). U momentu potpunog sazrevanja ploda, kazeinolitička aktivnost lateksa pada gotovo na polovinu inicijalne vrednosti (oko 40 U/mg) (slika 5.2A). Aktivnost prema kolagenu najintenzivnija je na početku perioda sazrevanja ploda i iznosi približno 105 CDU/mg, dok vremenom dolazi do postepenog opadanja ove aktivnosti, te uzorci lateksa iz avgusta pokazuju samo polovinu početne aktivnosti (oko 50 CDU/mg) (slika 5.2D).

5.2. Mapiranje po klasama proteaza lateksa smokve

U cilju mapiranja po klasama proteaza prisutnih u lateksu smokve, ispitivana je aktivnost lateksa prema supstratima u prisustvu različitih proteaznih inhibitora specifičnih za svaku od klasa proteaza. Rezultati datog eksperimenta prikazani su na slici 5.3.

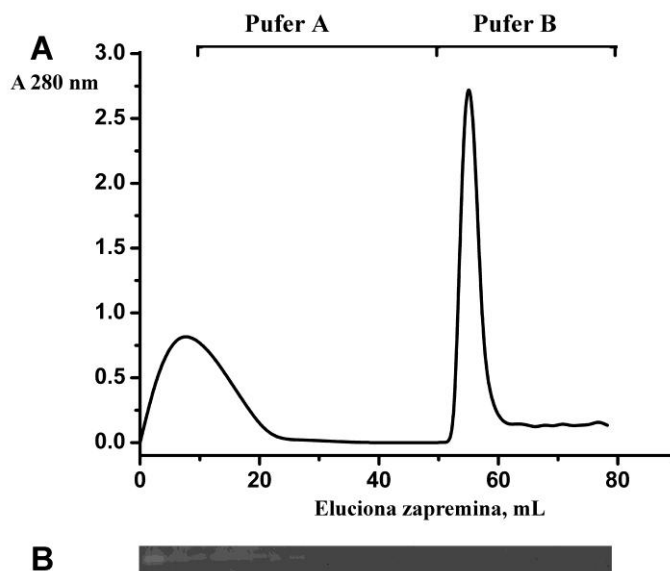


Slika 5.3. Efekat inhibitora na proteolitičke aktivnosti lateksa smokve. Rezidualna aktivnost je izračunata kao procenat enzimske aktivnosti u odnosu na neinhibirani uzorak lateksa. Prikazani rezultati predstavljaju srednje vrednosti \pm standardne devijacije rezultata dobijenih iz tri nezavisna eksperimenta.

Sa histogramskog prikaza efekata inhibitora na proteolitičku aktivnost lateksa smokve može se primetiti da korišćeni inhibitori različito utiču na aktivnosti prema četiri navedena supstrata. Pepstatin A ne utiče statistički značajno na aktivnosti prema bilo kom od navedenih supstrata, pošto je dobijena aktivnost nakon tretmana ovim inhibitorom nepromenjena u odnosu na neinhibirani uzorak lateksa i iznosi oko 100% (slika 5.3). S druge strane, IAA, E-64 i PMSF značajno utiču na aktivnost proteaza lateksa. Gotovo potpuna inhibicija aktivnosti prema supstratu BAPNA i kazeinu se dobija nakon tretmana lateksa inhibitorima IAA (10, odnosno 15% aktivnosti preostaje) i E-64 (2, odnosno 16% aktivnosti preostaje) (slika 5.3). Ova dva inhibitora, pak ne utiču na kolagenolitičku i želatinolitičku aktivnost. Primenom inhibitora PMSF dolazi do promena na nivou svih testiranih aktivnosti lateksa smokve. Najmanji procenat aktivnosti preostaje kada su kolagen i želatin korišćeni kao supstrati (5, odnosno 10%), dok u slučaju supstrata BAPNA i kazeina zaostaje 18, odnosno 38% (slika 5.3). U prisustvu inhibitora metaloproteaza, EDTA, dolazi do porasta aktivnosti prema svim supstratima i to do oko 18% (slika 5.3).

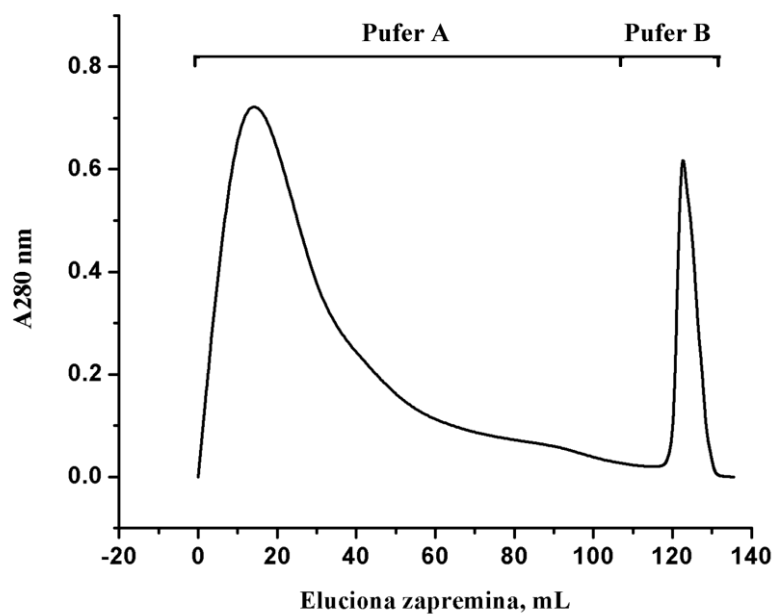
5.3. Optimizovanje postupka za paralelno izolovanje kolagenolitičke serin-proteaze i ficina

U cilju prečišćavanja dve predominantne klase proteaza lateksa smokve (cistein-proteaza – ficina i serin-proteaza), kako bi se ovi enzimi dalje pojedinačno okarakterisali, optimizovan je postupak prečišćavanja koji omogućava razdvajanje s jedne strane samo ficinskih formi, a sa druge strane samo kolagenolitičke serin-proteaze. Gel-filtracija na matriksu Sephadex G-75 nije bila dovoljno rezolutivna za prečišćavanje proteaza, ali je vodila eliminisanju proteina manjih molekulskih masa (<20 kDa), kao i većih molekulskih masa (>80 kDa). Frakcije koje su pokazivale želatinolitičku aktivnost u zimogramu spojene su i dalje razdvajane kovalentnom hromatografijom na matriksu tiol-Sepharose. Hromatogram je prikazan na slici 5.4.



Slika 5.4. A) Hromatografsko razdvajanje na tiol-Sepharose matriksu za kovalentnu hromatografiju. Kolona je ekvilibrisana i nevezani proteini eluirani 0,1 M Tris puferom pH 8,0 (pufer A), a potom su vezani proteini eluirani istim puferom koji sadrži 20 mM L-cistein (pufer B). Frakcije su sakupljene pod protokom od 0,8 mL/min. B) Aktivnost svake frakcije prema želatini određena je želatinskom SDS-PAGE.

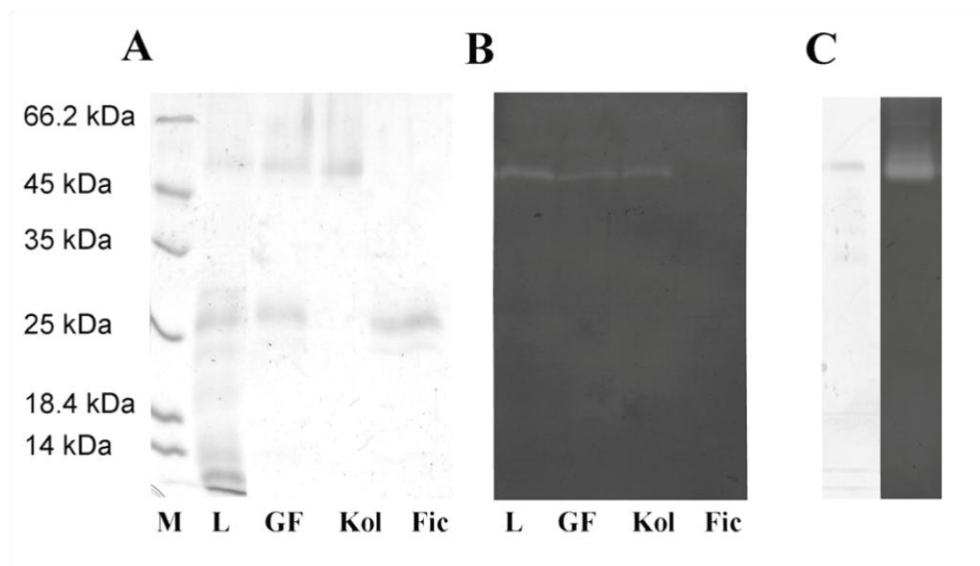
Sa slike 5.4. može se primetiti da se kovalentnom hromatografijom na matriksu sa sulfhidrilnim grupama dobija jasno razdvajanje cistein-proteaza od ostalih prisutnih proteina. Ostali proteini (frakcije nevezanih proteina) spojeni su i dalje razdvajani jonoizmenjivačkom hromatografijom na matriksu DEAE-Sepharose na pH 7,0, čime je prečišćena i kolagenolitička serin-proteaza. Hromatogram je prikazan na slici 5.5.



Slika 5.5. A) Jonoizmenjivačka hromatografija na DEAE-Sepharose matriksu. Kolona je ekvilibrisana i nevezani proteini eluirani 50 mM natrijum-fosfatnim puferom pH 7,0 (pufer A), a potom su vezani proteini eluirani povećanjem jonske sile, istim puferom koji sadrži 0,5 M natrijum-hlorid (pufer B). Frakcije su sakupljene pod protokom od 0,8 mL/min.

Dobija se jasno odvajanje proteina koji se vezuju za kolonu pri datim uslovima o čemu svedoči jasno odvojeni simetrični pik (slika 5.5).

Praćenje toka prečišćavanja ficina i kolagenolitičke serin-proteaze prikazano je na slici 5.6.



Slika 5.6. Elektroforetska analiza proteina lateksa smokve tokom procesa prečišćavanja: A) SDS-PAGE; B) zimogram nakon SDS-PAGE sa 0,1% želatinom inkorporiranim u gel za razdvajanje; L - lateks, GF - spojene proteolitičke frakcije eluirane sa kolone Sephadex G-75, Kol - kovalentna hromatografija - nevezani proteini, Fic - kovalentna hromatografija - vezani proteini, M - markeri molekulske mase; C) analiza prečišćene kolagenolitičke serin-proteaze nakon jonoizmenjivačke hromatografije SDS-PAGE elektroforezom i zimogramom.

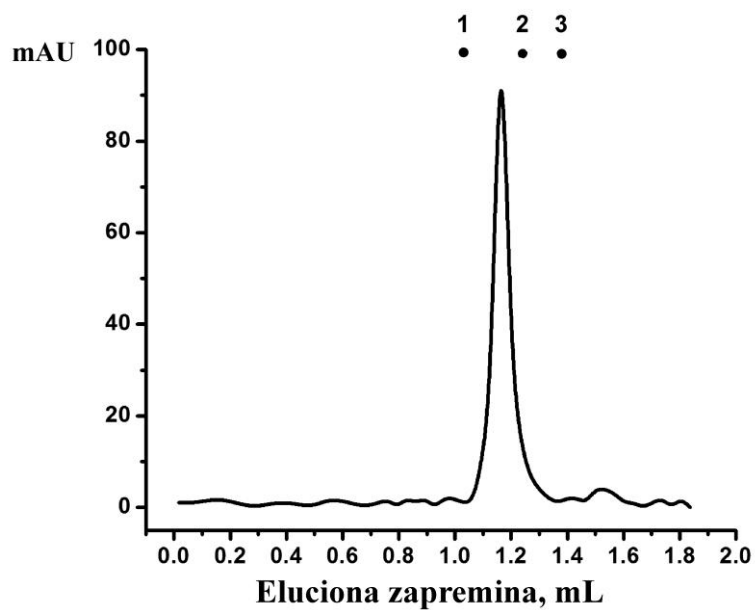
Sa slika 5.6A i 5.6B može se zapaziti da se nakon gel-filtracije i kovalentne hromatografije na tiol-Sepharose matriksu dobija homogena traka ficina (oko 25 kDa), dok se dodatnim korakom jonoizmenjivačke hromatografije dobija homogena traka kolagenaze (oko 46 kDa) na zimogramu i SDS-PAGE (slika 5.6C).

5.4. Biohemijska karakterizacija kolagenolitičke serin-proteaze i ficina izolovanih iz lateksa smokve

5.4.1. Biohemijska karakterizacija kolagenolitičke serin-proteaze lateksa smokve

5.4.1.1. Određivanje molekulske mase i pI vrednosti kolagenolitičke serin-proteaze

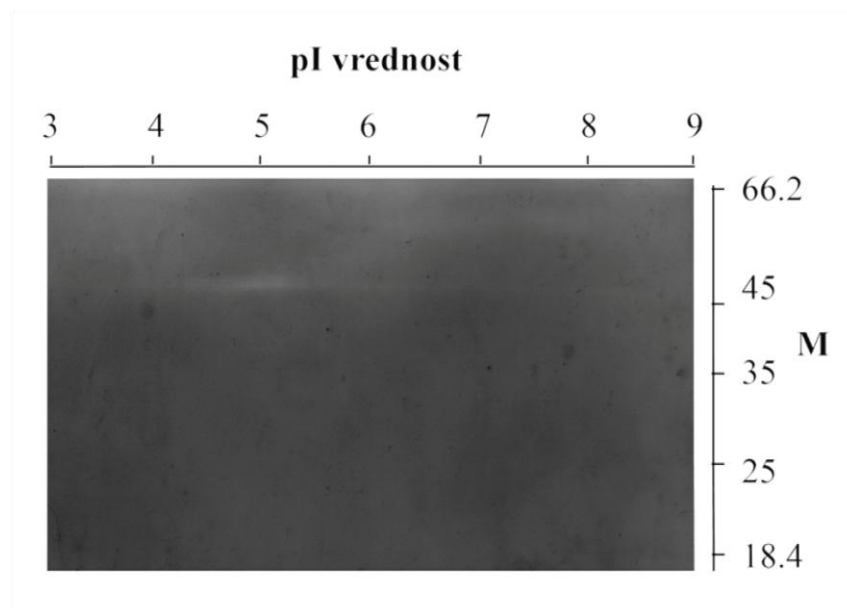
Karakterizacija prečišćene kolagenolitičke serin-proteaze najpre je podrazumevala određivanje molekulske mase proteina elektroforetski (slika 5.6A), kao i primenom analitičke gel-filtracije prečišćene kolagenolitičke serin-proteaze na matriksu Superdex 75 (slika 5.7).



Slika 5.7. Gel-filtracija prečišćene serin-proteaze lateksa smokve. Tačke 1, 2 i 3 - standardi molekulske mase (BSA, karboanhidraza, citohrom c, redom).

Kao rezultat analitičke gel-filtracije dobijen je simetričan pik koji odgovara molekulskoj masi od oko 41 kDa.

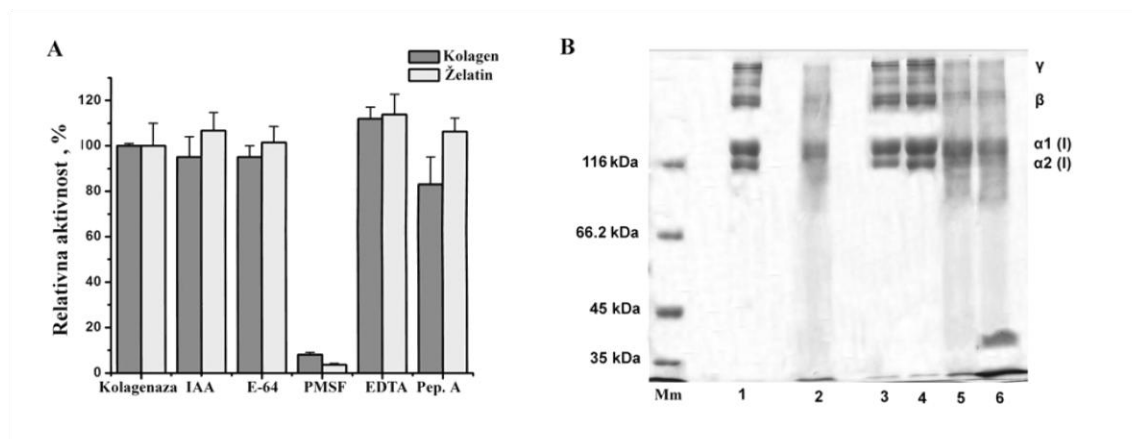
Masa (48 kDa) i pI vrednost (oko 5) prečišćene kolagenolitičke serin-proteaze određeni su 2D elektroforezom sa zimogramskom detekcijom nakon razdvajanja u drugoj dimenziji (slika 5.8).



Slika 5.8. Dvodimenzionalna SDS-PAGE prečišćene kolagenolitičke serin-proteaze. Izoelektrično fokusiranje u prvoj dimenziji i SDS-PAGE sa zimogramskom detekcijom nakon razdvajanja u drugoj dimenziji; M - markeri molekulske mase.

5.4.1.2. Određivanje supstratne specifičnosti kolagenolitičke serin-proteaze

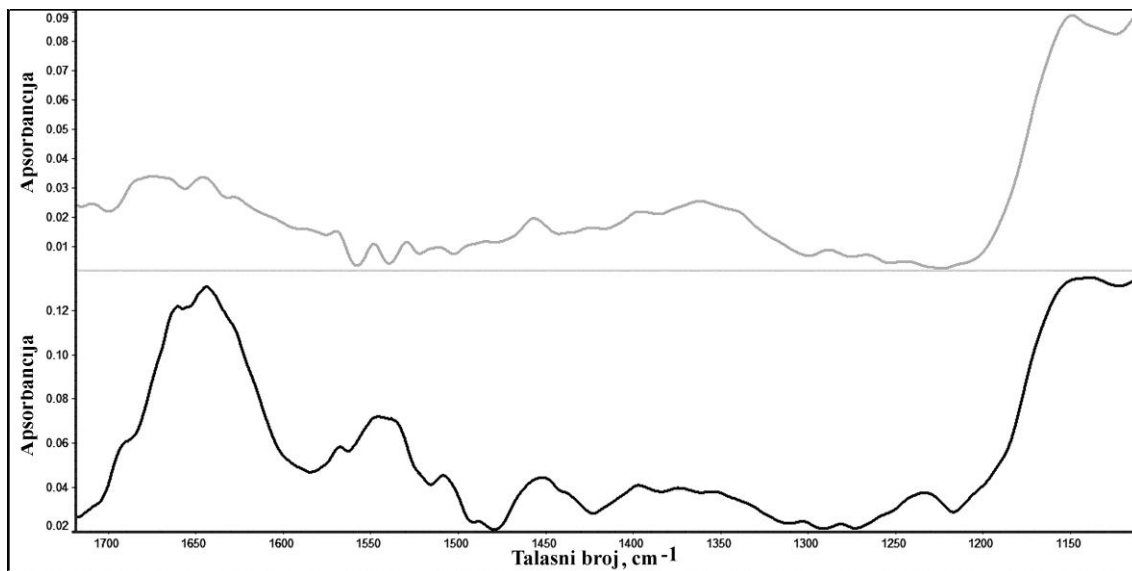
Kako veći broj proteina može da katalizuje hidrolizu želatina, dalji tok karakterizacije serin-proteaze išao je u smeru ispitivanja njene sposobnosti da hidrolizuje nativni kolagen. U te svrhe postavljena je hidroliza nativnog nerastvornog kolagena (slika 5.9A). Proizvodi digestije nativnog nerastvornog kolagena prikazani su na slici 5.9B.



Slika 5.9. Hidroliza želatina i kolagena tipa I prečišćenom serin-proteazom lateksa smokve i uticaj inhibitora na hidrolizu. A) Procenti preostale aktivnosti nakon inhibicije različitim supstratima u odnosu na neinhibirani enzim. Relativna aktivnost je određena ninhidrinskim testom hidrolize želatina i kolagena. IAA - jodoacetamid, E-64 - N-[N-(L-3-transkarboksiiran-2-karbonil)-L-Leucil]-agmatin, PMSF - fenilmetilsulfonil fluorid, EDTA - dinatrijumova so etilendiamin tetrasirćetne kiseline, Pep. A - pepstatin A. B) Hidroliza lanaca nativnog kolagena tipa I. Produkti digestije kolagena enzimom razdvojeni su na 7,5% gelu za razdvajanje primenom SDS-PAGE. 1 - kolagen (kontrola), 2 - neinhibirana serin-proteaza lateksa smokve sa kolagenom, 3, 4, 5 i 6 - digestija kolagena serin-proteazom koja je prethodno inhibirana različitim inhibitorima serin-proteaza, redom: PMSF, Pefabloc SC, benzamidin hidrohlorid i inhibitor tripsina iz soje. M - markeri molekulskih masa. $\alpha 1$ i $\alpha 2$ - pojedinačni lanci iz kojih je sastavljen kolagen tipa I, β - dimeri lanaca kolagena, γ - trimeri lanaca kolagena.

Na osnovu slike 5.9A može se zaključiti da prečišćena serin-proteaza lateksa smokve pored hidrolize želatina, ima i sposobnost hidrolize nativnog kolagena. Kolagenolitička serin-proteaza se u slučaju oba supstrata inhibira jedino PMSF-om, ireverzibilnim inhibitorom serin-proteaza. Ostali inhibitori ne utiču značajno na enzimsku aktivnost. Slika B prikazuje da se hidroliza kolagena odvija i na pojedinačnim lancima nativnog kolagena, jer se zapaža da, osim dimera i trimera, i $\alpha 1$ i $\alpha 2$ lanci bivaju degradovani. Upotreba različitih tipova inhibitora serin-proteaza pokazuje da PMSF i Pefabloc SC potpuno inhibiraju dejstvo serin-proteaze, dok benzamidin i inhibitor tripsina iz soje ne utiču na njenu aktivnost, jer se dobijaju proizvodi nalik na proizvode dobijene nakon digestije neinhibiranom kolagenazom.

U cilju nedvosmislene potvrde kolagenolitičke aktivnosti izolovane serin-proteaze, snimljeni su FT-IR spektri kolagena tipa I pre i nakon digestije serin-proteazom lateksa smokve (slika 5.10).



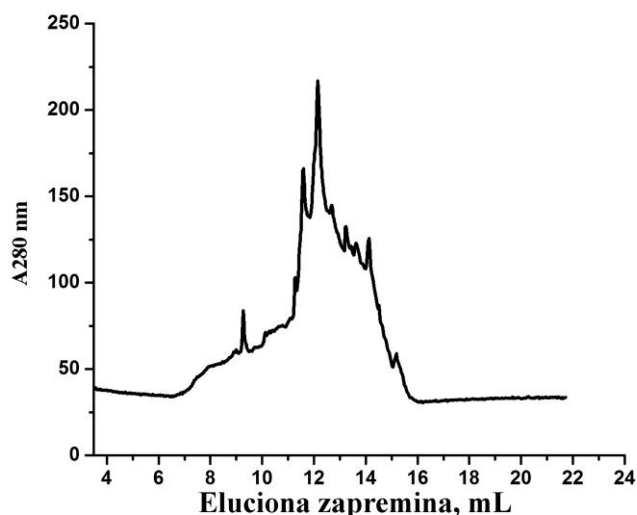
Slika 5.10. FT-IR spektar kolagena (kontrola) - crna linija; i kolagena tretiranog serin-proteazom lateksa smokve - siva linija.

Na slici 5.10 uočavaju se razlike između infracrvenih spektara nativnog i digestovanog kolagena u amidnom I ($1600-1700\text{ cm}^{-1}$), amidnom II ($1480-1575\text{ cm}^{-1}$) i amidnom III regionu ($1229-1301\text{ cm}^{-1}$) [142]. Naime, amidni I, II i III region, koji su veoma intenzivni u slučaju nativnog kolagena, nestaju nakon tretmana kolagenolitičkom serin-proteazom. Izračunat je odnos intenziteta trake amidnog III regiona na talasnom broju 1235 cm^{-1} i trake na talasnom broju 1450 cm^{-1} koja je karakteristična za heliks poliprolinskog tipa. Izračunat odnos intenziteta apsorbancija na karakterističnim talasnim brojevima (A^{1235}/A^{1450}) u slučaju nativnog kolagena iznosio je 0,91, dok je za digestovani kolagen bio 0,21, što ukazuje da je struktura trostrukog heliksa narušena ($<0,5$ [80]).

Serin-proteaza lateksa smokve okarakterisana je i u kontekstu temperaturne i pH stabilnosti i optimuma. Rezultati su prikazani u okviru priloga 1.

5.4.2. Biohemijska karakterizacija cistein-proteaza lateksa smokve - ficina

Izolovani ficin analiziran je reverzno-faznom hromatografijom u cilju potvrde prisustva više različitih izoformi. Hromatogram je prikazan na slici 5.11.



Slika 5.11. RP-HPLC hromatogram prečišćenog ficina lateksa smokve.

Analizom prečišćenog ficina reverzno-faznom hromatografijom dobijen je veći broj pikova od kojih je većina eluirana u relativno maloj retencionalnoj zapremini (12-16 mL) (slika 5.11).

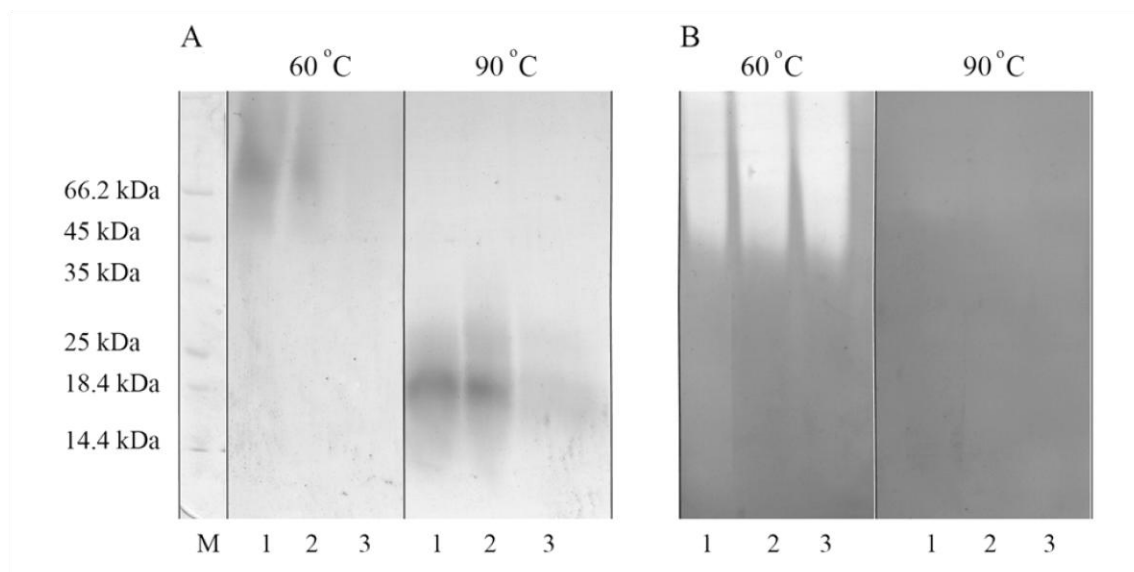
5.5. Prečišćavanje cistein proteaze – papaina

5.5.1. Kovalentna hromatografija

Papain je prečišćen kovalentnom hromatografijom na matriksu tiol-Sepharose iz komercijalnog preparata u cilju uklanjanja neaktivnog/nenativnog proteina i niskomolekulskih pigmenata prisutnih u preparatu. Na ovaj način papain je prečišćen 8 puta. Tabela praćenja toka prečišćavanja, kao i hromatogram dati su u prilogu (prilog 2 i 3).

5.5.2. Elektroforeza/zimogram i identifikacija papaina

Prečišćavanje i aktivnost papaina praćeni su i elektroforetski, odnosno zimogramom. Rezultati su prikazani na slici 5.12.



Slika 5.12. A) SDS-PAGE analiza prečišćavanja papaina i B) zimogram sa 0,1% želatinom (inkorporiranim u gel za razdvajanje): 1 – početni ekstrakt komercijalnog preparata papaina; 2 – frakcija papaina nakon taloženja acetonom; 3 – vezani protein nakon hromatografije na matriksu tiol-Sepharose; M – markeri molekulske mase.

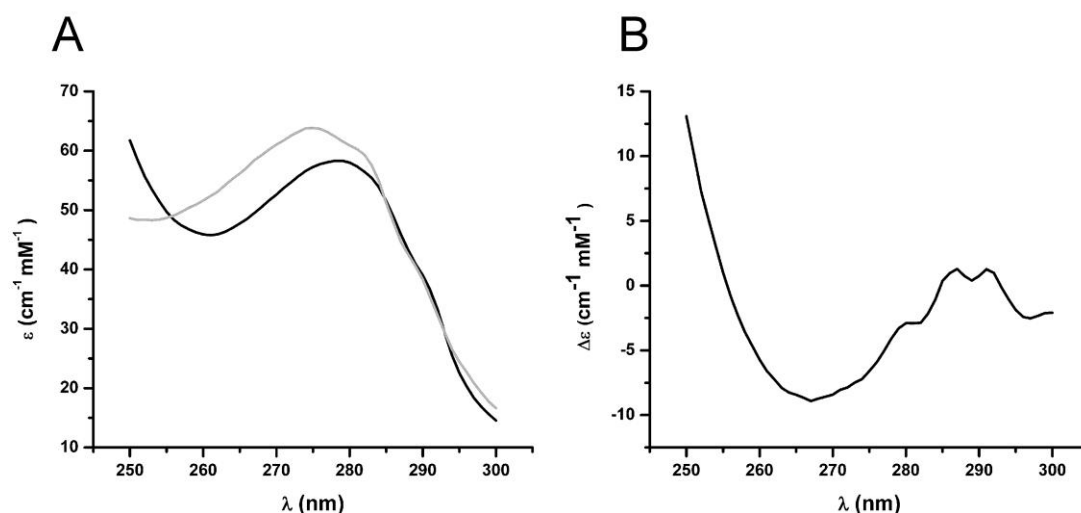
Primećuje se znatno sporija migracija uzoraka inkubiranih na 60 °C u poređenju sa uzorcima inkubiranim na 95 °C (slika 5.12A). Uzorci inkubirani na 60 °C zadržali su svoju aktivnost u zimogramu, dok nakon inkubacije na 95 °C papain biva potpuno inaktiviran (slika 5.12B).

Nakon SDS-PAGE i bojenja rastvorom boje CBB, izolovani protein identifikovan je masenom spektrometrijom peptida dobijenih nakon digestije tripsinom (*engl.* TMF – trypsin mass fingerprinting) (prilog 4).

5.6. Optimizovanje metodologije za ispitivanje stabilnosti proteaza na niskim temperaturama

5.6.1. Ispitivanje termalne denaturacije papaina diferencijalnom UV spektroskopijom u širokom temperaturnom intervalu

Nativni i termalno denaturisani papain upoređeni su snimanjem UV spektara na talasnim dužinama od 250 do 300 nm (slika 5.13).

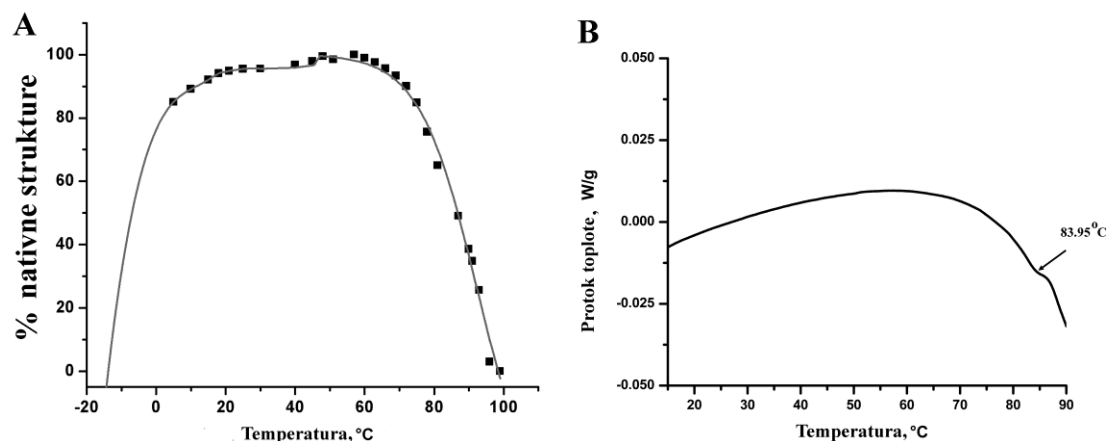


Slika 5.13. A) UV apsorpcioni spektar od 250 nm do 300 nm nativnog (crna linija) i denaturisanog papaina (siva linija); B) Diferencijalni UV apsorpcioni spektar.

Na UV spektru papaina očigledna je razlika između apsorpcije nativnog i denaturisanog molekula papaina. Maksimalni ekstinkcioni koeficijent nativnog molekula papaina izmeren je na 279 nm ($58,332 \text{ cm}^{-1} \text{ mM}^{-1}$), dok je maksimum u slučaju denaturisanog papaina pomeren ka nižim vrednostima talasne dužine, tačnije nalazi se na 275 nm ($63,855 \text{ cm}^{-1} \text{ mM}^{-1}$) (slika 5.13A).

Da bi razlike između spektara nativnog i denaturisanog papaina bile uočljivije, izračunat je i diferencijalni UV spektar u istoj oblasti (slika 5.13B). Računanjem diferencijalnog spektra omogućava se određivanje talasne dužine na kojoj postoji najizraženija razlika između dva spektra. Najveća razlika u ekstinkcionim koeficijentima nativnog i denaturisanog papaina postoji na 267 nm i iznosi $-8,912 \text{ cm}^{-1} \text{ mM}^{-1}$. Iako najosetljivija, oblast ispod 260 nm nije odabrana zbog nekoherentnosti rezultata merenja u narednim eksperimentima.

Na osnovu prethodnog rezultata, talasna dužina od 267 nm izabrana je za praćenje zavisnosti apsorpcije od temperature, na osnovu čega je bilo moguće konstruisati krivu termalne denaturacije papaina. Usled stvaranja leda na nižim temperaturama, praćenje apsorpcije bilo je moguće u temperaturnom intervalu 5-99 °C (slika 5.14).



Slika 5.14. A) Kriva termalne denaturacije papaina inkubiranog na sniženim i povišenim temperaturama. Praćena je apsorbancija na 267 nm u funkciji temperature u odgovarajućem temperaturnom intervalu (5-99 °C). Brzina zagrevanja je iznosila 0,5 °C/min. Procenat native strukture je izračunat je kao procenat maksimalne razlike ekstinkcionih koeficijenata ($\Delta\epsilon$); B) DSC termogram papaina u 50 mM Tris puferu pH 8,0 (temperaturni interval 10-90 °C).

Sa krive termalne denaturacije (slika 5.14A) uočava se da je papain najstabilniji u temperaturnom intervalu od 45 °C do 60 °C sa maksimalnom stabilnošću na 57 °C. Porastom temperature sa 60 °C na 95 °C dolazi do pada procenta native strukture sa 99,5% na samo 0,5%. Na niskoj temperaturi, u delu krive denaturacije ispod 45 °C može se takođe zapaziti redukcija procenta native strukture; na 5 °C procenat native strukture snižen je za 18%.

Određena vrednost temperature termalne denaturacije u regionu povišene temperature (T_m) iznosila je 84 ± 1 °C (slika 14A), odnosno 83,95 °C (vrednost određena DSC metodom) (slika 5.14B). Termogram takođe pokazuje blagi pad protoka toplote na temperaturama ispod 40 °C.

Određena je vrednost temperature termalne denaturacije u regionu snižene temperature (T_c) sa ekstrapoliranog dela krive i iznosila je -7 ± 1 °C (slika 14A).

Na osnovu dobijenih rezultata praćenja promene apsorbancije u zavisnosti od temperature, izračunati su i termodinamički parametri procesa denaturacije, promena Gibsove slobodne energije papaina (ΔG°) na 5 °C, 25 °C, 48 °C i 60 °C. Rezultati su prikazani u okviru tabele 5.1.

Tabela 5.1. Gibsova slobodna energija (ΔG°) termalne stabilnosti papaina. ΔG° parametar je izračunat iz jednačine $\Delta G^\circ = -RT \ln K$ gde je R univerzalna gasna konstanta, a T apsolutna temperatura.

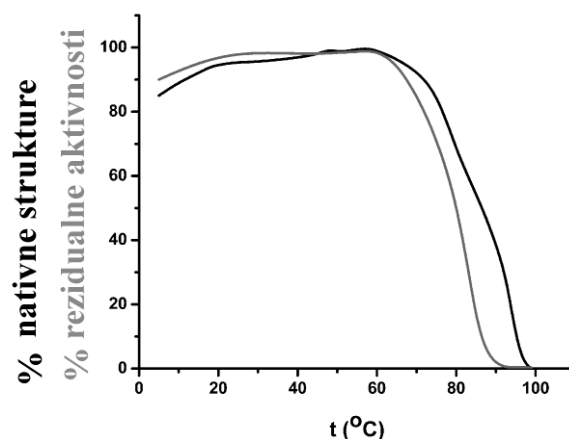
	ΔG°_{278} (kJ/mol)	ΔG°_{298} (kJ/mol)	ΔG°_{321} (kJ/mol)	ΔG°_{333} (kJ/mol)
Papain	$3,2 \pm 0,1$	$7,5 \pm 0,3$	$13,9 \pm 0,3$	$12,5 \pm 0,4$

Na 48 °C dobijena je najviša vrednost Gibsove slobodne energije za papain ($13,9 \pm 0,3$ kJ/mol) (tabela 5.1).

5.6.2. Ispitivanje kinetičke stabilnosti papaina

5.6.2.1. Određivanje brzina inaktivacije papaina

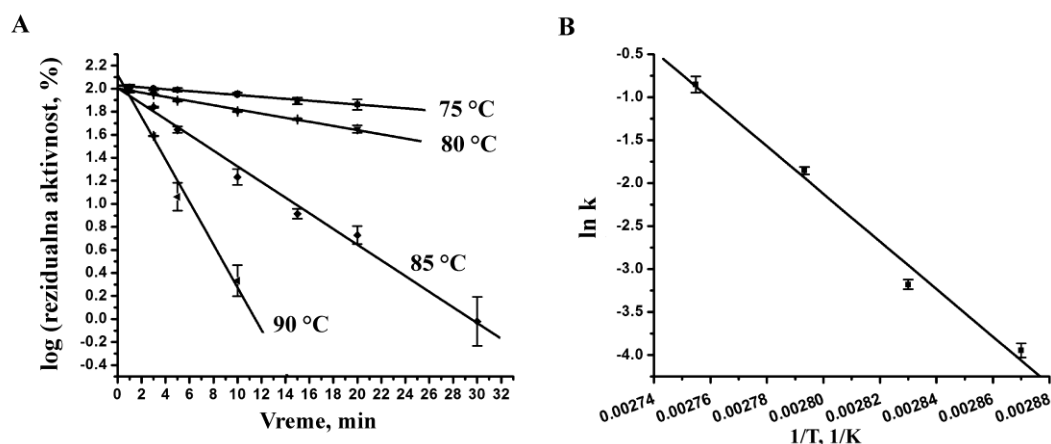
Budući da se papain smatra kinetički stabilnim proteinom [143], dalji cilj ovog istraživanja bila je potvrda kinetičke stabilnosti papaina u prisustvu detergenta (prilog 5) i na povišenim temperaturama uz predlaganje strukturnog objašnjenja kinetičke stabilnosti koja se dovodi u vezu i sa otpornošću kinetički stabilnih proteina na denaturaciju. U cilju ispitivanja zavisnosti inaktivacije papaina od temperature, određena je rezidualna aktivnost papaina na različitim temperaturama korišćenjem supstrata BAPNA. U temperaturnom intervalu od 5 do 100 °C konstruisana je kriva inaktivacije papaina (slika 5.15).



Slika 5.15. Krive termalne denaturacije (crna linija) i termalne inaktivacije (siva linija) papaina. Kriva termalne inaktivacije je konstruisana korišćenjem supstrata BAPNA i praćenjem apsorpcije na 405 nm u funkciji od temperature. Rezidualna aktivnost je izračunata kao procenat maksimalne aktivnosti određene na 40 °C.

Kriva termalne inaktivacije opada ranije u odnosu na krivu denaturacije papaina (slika 5.15). Izračunata je vrednost temperature na kojoj je 50% papaina inaktivirano (T_{m50} vrednost) koja iznosi 79 ± 2 °C, u odnosu na T_m vrednost na kojoj je 50% papaina denaturisano koja iznosi 84 ± 1 °C.

Za temperaturne tačke u opadajućem delu krive određene su konstante brzina inaktivacije. Eksponencijalne krive prvog reda zavisnosti rezidualne aktivnosti enzima od vremena inkubacije na datim temperaturama su semilogaritamski linearizovane i rezultati linearizacije prikazani na slici 5.16A. Konstante brzina inaktivacije izračunate su iz vrednosti nagiba linearnom regresionom analizom (tabela 5.2).



Slika 5.16. A) Termalna inaktivacija papaina u opsegu temperatura od 75 do 90 °C; B) Arenijusov grafik koji prikazuje temperaturnu zavisnost konstante brzine inaktivacije papaina.

Tabela 5.2. Konstante brzina inaktivacije (k , min⁻¹) papaina na različitim temperaturama.

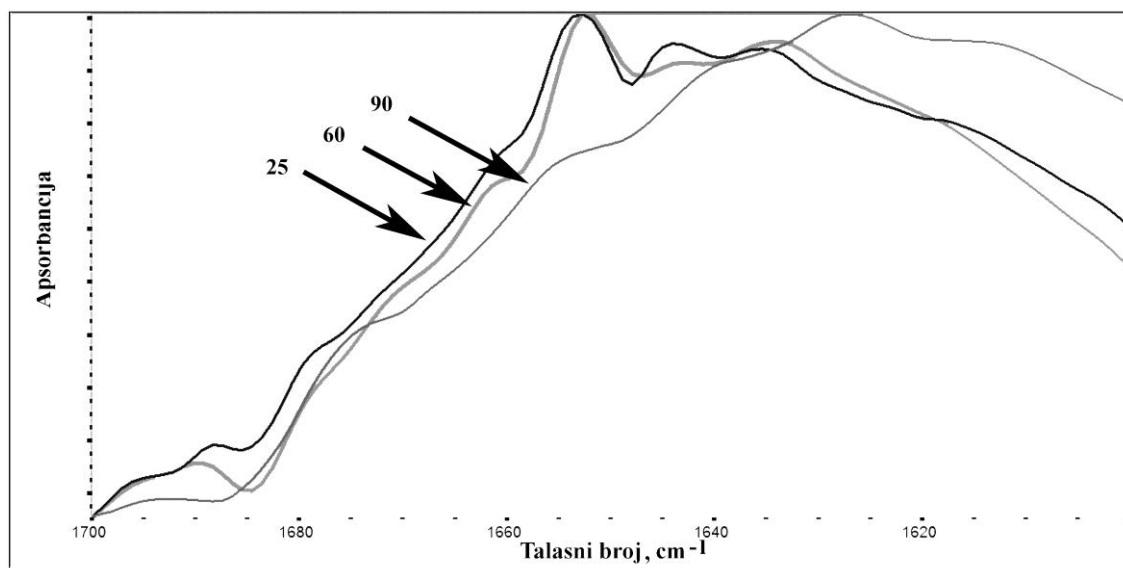
T, °C	75	80	85	90
Papain k , min ⁻¹	0,019 ±0,002	0,042 ±0,002	0,157 ±0,007	0,43 ±0,04

U temperaturnom opsegu od 75 °C do 90 °C dolazi do povećanja konstante brzine inaktivacije papaina sa početnih 0,019 min⁻¹ na čak 0,43 min⁻¹ na 90 °C (tabela 5.2).

Na osnovu Arenijusovog grafika (slika 5.16B) izračunata je vrednost aktivacione energije inaktivacije papaina koja je iznosila 223 ± 6 kJ/mol.

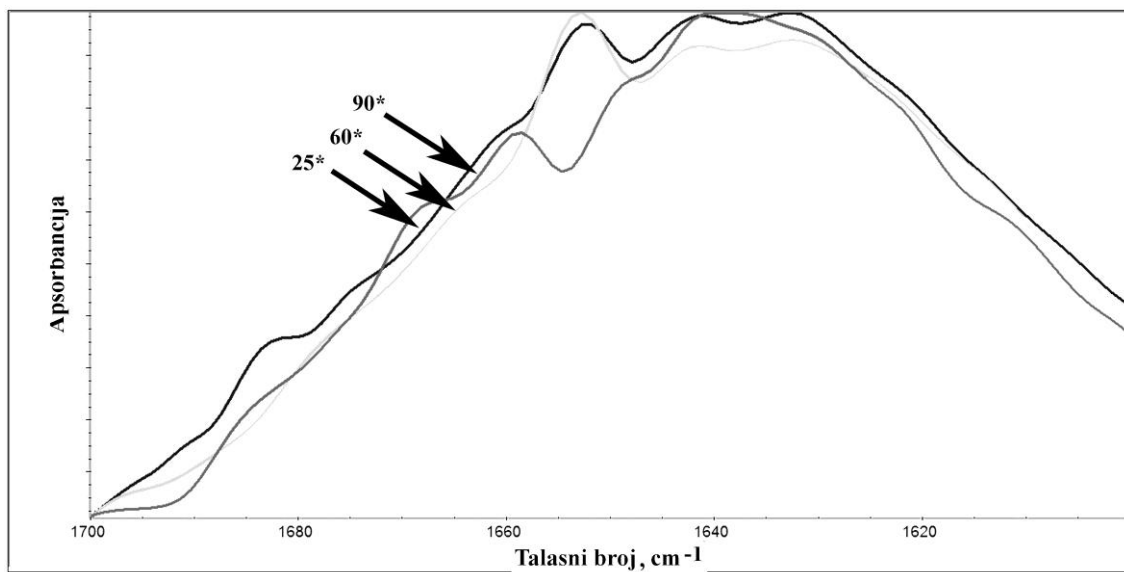
5.6.2.2. Strukturne promene pri denaturaciji papaina na visokim temperaturama i u prisustvu SDS

Snimanjem FT-IR spektara uzoraka papaina inkubiranih na različitim temperaturama sa i bez prisustva SDS-a dobijeni su podaci o promenama koje se dešavaju na nivou sekundarnih struktura papaina. Rezultati FT-IR spektroskopije prikazani su na slikama 5.17 i 5.18.



Slika 5.17. FT-IR spektri obrađeni metodom Furijeove samodekonvolucije, papaina inkubiranog na 25, 60 i 90 °C.

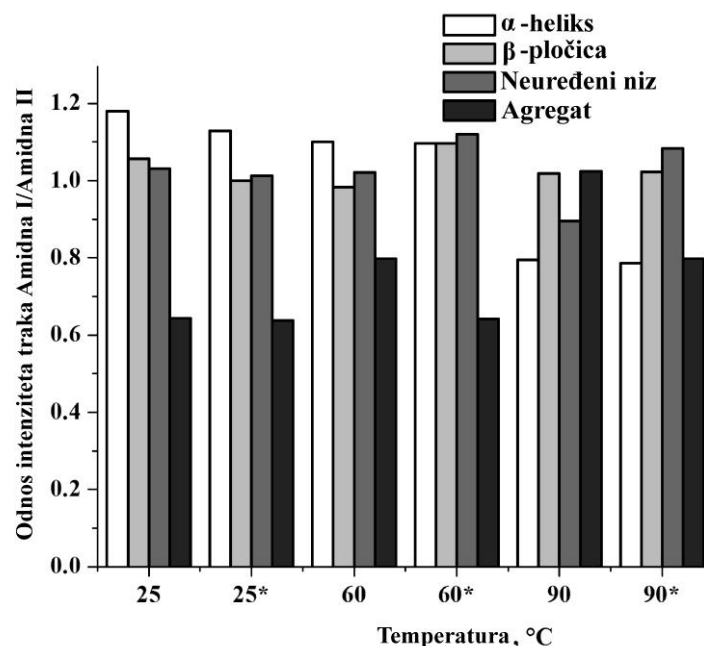
Inkubiranje papaina na povišenoj temperaturi (90 °C) dovelo je do smanjenja intenziteta trake na 1654 cm⁻¹ i 1643 cm⁻¹ (slika 5.17). S druge strane, primećuje se da dolazi do povećavanja apsorbanije u regionu na talasnom broju 1612 cm⁻¹ papaina inkubiranog na 90 °C.



Slika 5.18. FT-IR spektri obrađeni metodom Furijeove samodekonvolucije, papaina inkubiranog na 25, 60 i 90 °C u prisustvu 1% SDS-a.

Kada je papain inkubiran u prisustvu SDS-a, dobijeni su nešto drugačiji spektri. Na slici 5.18 primećuje se da je apsorpcija u regionu oko 1612 cm^{-1} u prisustvu SDS-a, čak i na 90 °C, prilično smanjena. Rezultati još ukazuju i na očuvanje trake na 1643 cm^{-1} i povećanje intenziteta trake na 1659 cm^{-1} . Takođe, i ovde dolazi do smanjenja intenziteta trake na 1654 cm^{-1} sa porastom temperature (slika 5.18).

U cilju praćenja sklonosti za zauzimanje određene sekundarne strukture u različitim uslovima, promene u odnosima intenziteta traka specifičnih za određene sekundarne strukture u amidnom I regionu i amidne II trake (kao internog standarda) [132, 144] takođe su prikazani histogramski (slika 5.19).



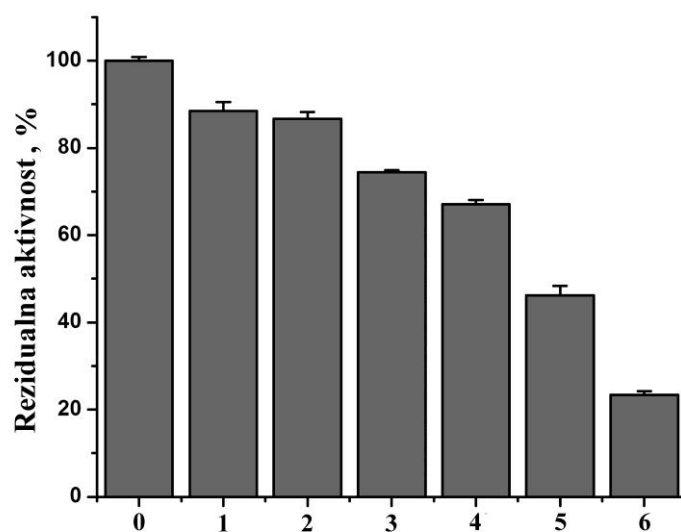
Slika 5.19. Promene u odnosu intenziteta traka karakterističnih za određene sekundarne strukture u IR spektru papaina inkubiranog na 25, 60 i 90 °C i uz dodatak 1% SDS-a (25*, 60* i 90*). Odnosi intenziteta amidna I traka/amidna II traka izračunati su korišćenjem traka specifičnih za sekundarne strukture u amidnom I regionu (1654 cm^{-1} α -heliks; 1632 cm^{-1} β -pločica; 1643 cm^{-1} neuređeni niz; 1612 cm^{-1} intermolekulska β -pločica specifična za agregaciju), i trake amidnog II regiona maksimalnog intenziteta (1520 cm^{-1}).

U papainu inkubiranom na 60 °C ne uočavaju se dramatične promene strukture (bez obzira na prisustvo ili odsustvo detergenta). Sa druge strane, u papainu inkubiranom na 90 °C, uočava se tendencija pada sadržaja α -heliksa i neuređenog niza u korist agregacionih β -pločica, uz očuvanje nativnih β -strukture. Pad sadržaja α -heliksa detektovan je na istoj temperaturi i u prisustvu detergenta, uz porast sadržaja neuređenog niza.

5.7. Ispitivanje stabilnosti papaina na niskim temperaturama

5.7.1. Ispitivanje aktivnosti papaina na niskim temperaturama

Aktivnost papaina tokom ponovljenih ciklusa inkubiranja na -20 °C praćena je korišćenjem supstrata BAPNA. Merena je rezidualna aktivnost nakon svakog od 6 ciklusa zamrzavanja i odmrzavanja. Rezultati praćenja enzimske aktivnosti papaina prikazani su na slici 5.20.

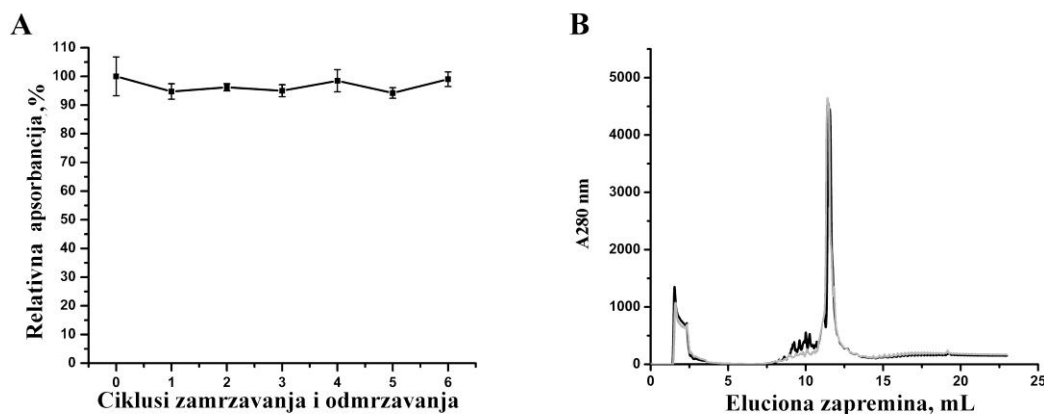


Slika 5.20. Aktivnost papaina tokom šest ciklusa zamrzavanja/odmrzavanja. Aktivnost startnog uzorka papaina (0) posmatrana je kao 100%, dok su izmerene aktivnosti nakon svakog ciklusa (1-6) izražene kao procenti startne aktivnosti. Prikazani rezultati predstavljaju srednje vrednosti \pm standardne devijacije rezultata dobijenih iz tri nezavisna eksperimenta.

Nakon svakog ciklusa zapaža se postepen pad aktivnosti papaina za oko 5-20%, čime nakon 6. ciklusa zamrzavanja/odmrzavanja, aktivnost papaina biva smanjena za čak 75% (slika 5.20).

5.7.2. Ispitivanje stabilnosti primarne strukture papaina na niskim temperaturama

Autoproteoliza papaina tokom šest ciklusa zamrzavanja/odmrzavanja praćena je testom vezivanja boje CBB. Rezultati su prikazani na slici 5.21A. S druge strane, u cilju detektovanja eventualnih promena u primarnoj strukturi papaina usled autoproteolize, urađena je i reverzno-fazna hromatografija (RP-HPLC) (slika 5.21B).

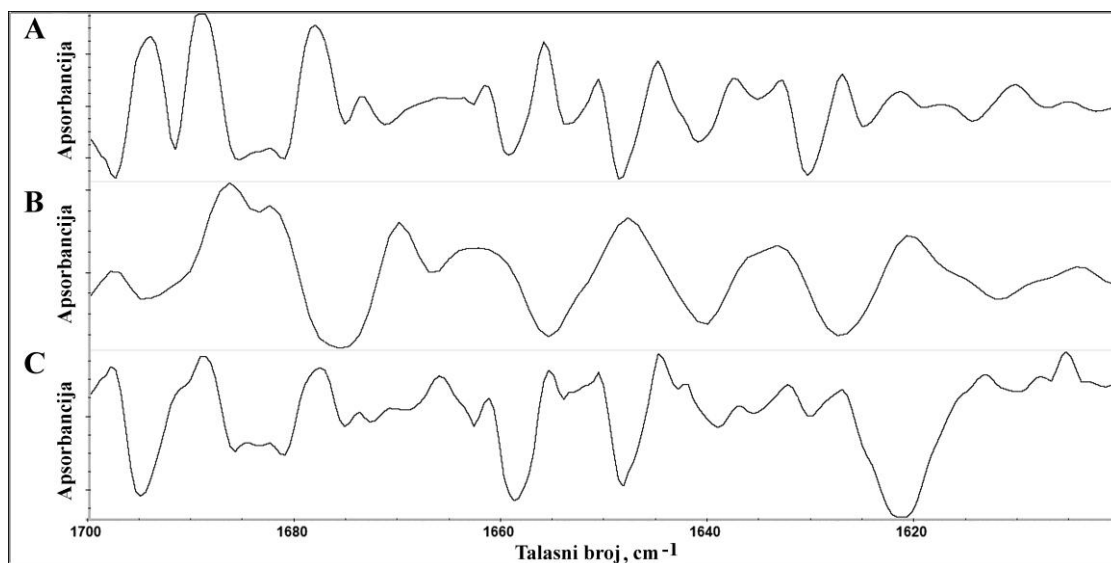


Slika 5.21. A) Autoproteoliza papaina tokom šest ciklusa zamrzavanja/odmrzavanja. Apsorbancija nakon svakog ciklusa izražena je kao procenat inicijalne apsorbancije; **B)** RP-HPLC nativnog papaina (crna linija) i papaina čuvanog na niskoj temperaturi (siva linija).

Sa slike 5.21A primećuje se da ne dolazi do smanjenja vezivanja boje CBB za papain jer je merena vrednost apsorbancije na 620 nm tokom 6 ciklusa zamrzavanja/odmrzavanja ostala na nivou od 100% (u okviru greške merenja). Takođe, pre i posle 6 ciklusa zamrzavanja/odmrzavanja, papain se eluiira sa reverzno-fazne kolone na istoj retencionalnoj zapremini (11,5 mL) kao jedan pik istog intenziteta (slika 5.21B).

5.7.3. Ispitivanje sekundarnih struktura papaina na niskim temperaturama

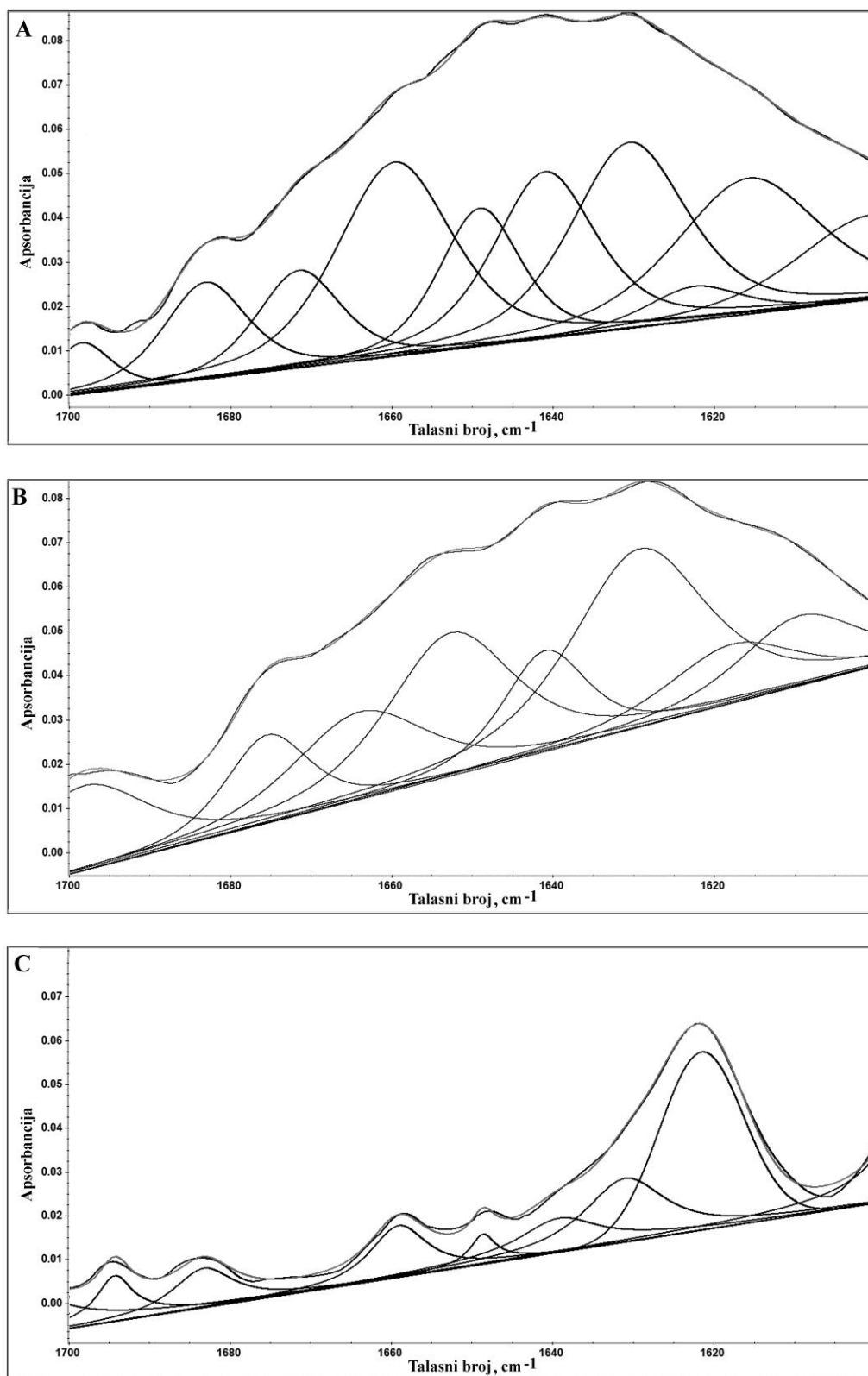
Infracrveni spektri sa Furijeovom transformacijom snimljeni su za nativni papain, kao i za papain inkubiran na visokoj i niskoj temperaturi. Promene u drugom izvodu spektara amidnog I regiona prikazane su na slici 5.22.



Slika 5.22. Drugi izvodi FT-IR spektara: A) nativnog papaina; B) papaina inkubiranog na visokoj temperaturi i C) papaina inkubiranog na niskoj temperaturi.

Sa prikaza drugog izvoda spektra nativnog papaina može se uočiti nekoliko traka (minimuma), naročito u regionu koji se dodeljuje uređenim sekundarnim strukturama. Najdominantniji minimumi prisutni su na sledećim talasnim brojevima: 1630, 1641, 1648, 1659, 1672, kao i oko 1683 i 1697 cm^{-1} (slika 5.22A). Najizraženija razlika u drugom izvodu spektra papaina inkubiranog na visokoj temperaturi (slika 5.22B) jeste gubitak trake na 1648 cm^{-1} , koja se pripisuje α -heliksu [135]. Drugi izvod spektra papaina inkubiranog na niskoj temperaturi razlikovao se od spektra nativnog papaina po tome što je najizraženija traka pomerena na 1623 cm^{-1} ukazujući na formiranje intermolekulske β -pločice (slika 5.22C) [56, 137].

Nakon razlaganja FT-IR spektara na komponente (slika 5.23), pikovi su dodeljeni specifičnim sekundarnim strukturama (tabela 5.3) i procenat svake sekundarne strukture izračunat je na osnovu površina ispod karakterističnih pikova u odnosu na površinu čitavog amidnog I regiona. Sadržaj sekundarnih struktura izračunat je i iz trodimenzionalne strukture određene difrakcijom X-zraka iz UniProt baze podataka za ulaz P00784 (PAPA1_CARPA). Rezultati su prikazani u tabeli 5.4.



Slika 5.23. Originalni i fitovani FT-IR spektar: A) nativnog papaina; B) papaina inkubiranog na visokoj temperaturi i C) papaina inkubiranog na niskoj temperaturi (nakon 6 ciklusa zamrzavanje/odmrzavanje).

Tabela 5.3. Dodeljivanje traka amidnog I regiona karakterističnim sekundarnim strukturama i relativne površine ispod traka u odnosu na kompletni amidni I region u spektrima nativnog papaina, papaina inkubiranog na visokoj temperaturi i papaina inkubiranog na niskoj temperaturi (nakon 6 ciklusa zamrzavanje/odmrzavanje). Relativne površine ispod traka (u procentima) određene su metodom fitovanja u krivu. Inter - intermolekulska β -pločica; intra - intramolekulska β -pločica.

Nativni Papain		Papain inkubiran na visokoj temperaturi		Papain inkubiran na niskoj temperaturi		Dodeljena sekundarna struktura
ν (cm^{-1})	Površina (%)	ν (cm^{-1})	Površina (%)	ν (cm^{-1})	Površina (%)	
1623	3,6	1618	9,1	1623	38,7	Inter/Intra β [56, 135-137]
1630	22,8	1628	27,2	1629	19,2	Inter β -pločica [135]
1641	19,8	1641	9,7	1639	11,7	Neuređeni niz [135]
1648	10,8	-	-	1648	3,4	α -heliks [135]
1659	21,3	1653	20,6	1659	10,7	α -heliks [135]
1672	9,9	1674	8,2	-	-	Neuređeni heliks [136]
1683	8,5	1663	13,0	1683	8,8	Zavijutak [135]
1697	3,3	1698	12,2	1695	7,5	Inter/Intra β [56, 135, 136]

Tabela 5.4. Sadržaj sekundarnih struktura (%) dobijen fitovanjem u krivu prisutan u nativnom papainu, papainu inkubiranom na visokoj temperaturi i papainu inkubiranom na niskoj temperaturi (nakon 6 ciklusa zamrzavanje/odmrzavanje). Rezultati su dobijeni primenom ATR FT-IR spektroskopije, ali je sadržaj sekundarnih struktura izračunat i iz podataka dobijenih difrakcijom X-zraka.

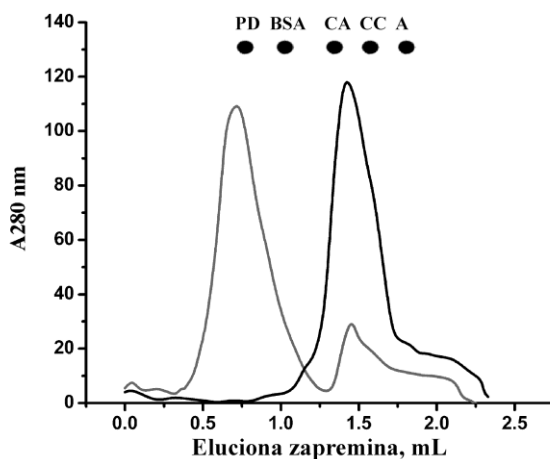
Uzorak papaina	Sadržaj sekundarnih struktura (%)					
	β -pločica	α -heliks	Zavijutak	Neuređene strukture	Inter/Intra β -pločica	Greška
Nativni papain	23,0	32,2	8,6	30,0	6,2	3,5
Papain inkubiran na visokoj temperaturi	27,2	20,6	13,0	17,9	21,3	0,1
Papain inkubiran na niskoj temperaturi	19,2	11,7	8,8	14,1	46,2	1,4
Difrakcija X-zraka	25	26	7	42	-	-

Upoređivanje sadržaja sekundarnih struktura nativnog papaina i papaina inkubiranog na visokoj temperaturi, ukazuje da nakon inkubacije na visokoj temperaturi dolazi do porasta sadržaja β -pločica i to naročito na račun α -heliksa i neuređenih struktura (tabela 5.4). Najizraženije promene u sadržaju sekundarnih struktura indukovane su inkubacijom papaina na niskim temperaturama (slika 5.23, tabela 5.4). Šest uzastopnih ciklusa zamrzavanja/odmrzavanja papaina indukuje pad u procentu α -helikoidne

strukture (sa 32,2% kod nativnog papaina na 11,7%) (slika 5.23C). Najizrazitija promena je porast sadržaja intermolekulskih β -pločica koje su specifične za agregiranje proteina [56, 137] (sa svega 6,2% inter/intramolekulske β -pločice kod nativnog papaina na čak 46,2% kod papaina podvrgnutog uzastopnim ciklusima zamrzavanja/odmrzavanja).

5.7.4. Ispitivanje agregiranja papaina tokom izlaganja niskim temperaturama

Kako su rezultati promena sekundarnih struktura indicirali da dolazi do stvaranja agregata papaina tokom izlaganja niskim temperaturama, agregiranje papaina praćeno je gel-filtracijom visokih performansi. Dobijeni hromatogram prikazan je na slici 5.24.



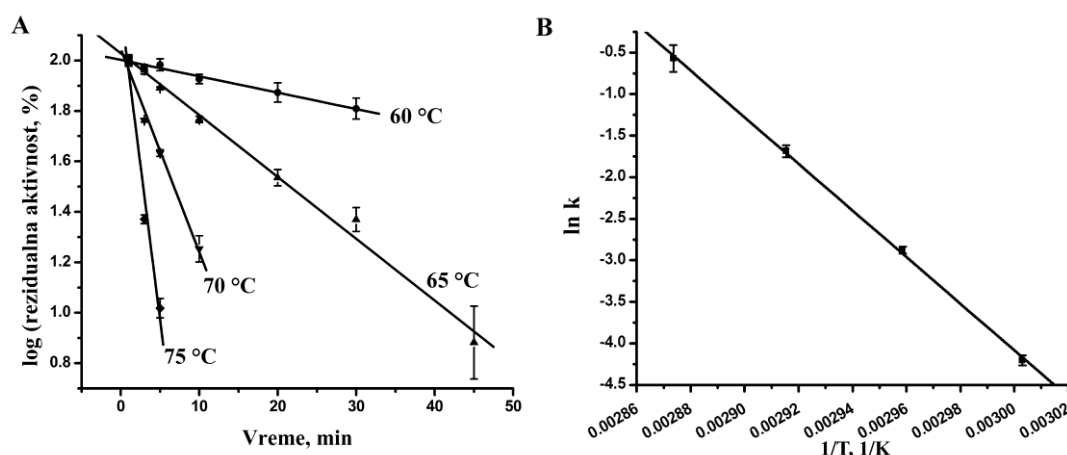
Slika 5.24. Gel-filtracija nativnog papaina (crna linija) i papaina inkubiranog na niskoj temperaturi (nakon 6 ciklusa zamrzavanje/odmrzavanje) (siva linija). PD, BSA, CA, CC i A - standardi molekulskih masa (PD - plavi dekstran 2000 kDa, BSA - goveđi serum albumin 67 kDa, CA - karbonska anhidraza 29 kDa, CC - citohrom c 14 kDa i A - aprotinin 6,5 kDa).

Hromatogram nativnog papaina pokazuje jedan dominantni pik koji odgovara izračunatoj masi od 24,0 kDa. Na hromatogramu papaina koji je izlagan uzastopnim ciklusima zamrzavanja/odmrzavanja pik koji odgovara nativnom papainu znatno je manjeg intenziteta, dok se oko 90% papaina iz ovog uzorka eluiralo u mrtvoj zapremini kolone (slika 5.24).

5.8. Ispitivanje termalne stabilnosti ficina

5.8.1. Ispitivanje kinetičke stabilnosti ficina

Na osnovu strukturne sličnosti između ficina i papaina, kao i na osnovu prethodno zapažene rezistencije ficina na SDS, pretpostavljeno je da i ficin potencijalno predstavlja jedan od kinetički stabilnih proteina. U cilju ispitivanja kinetičke stabilnosti ficina, konstruisana je kriva termalne inaktivacije ficina, kao i Arenijusova zavisnost (slika 5.25).



Slika 5.25. A) Termalna inaktivacija ficina u opsegu temperatura od 60 do 75 °C; B) Arenijusova zavisnost koji prikazuje temperaturnu zavisnost konstante brzine inaktivacije ficina.

Konstante brzina inaktivacije izračunate su na osnovu nagiba dobijenih linearnom regresionom analizom i prikazane u tabeli 5.5.

Tabela 5.5. Konstante brzina inaktivacije (k , min^{-1}) ficina na različitim temperaturama.

T, °C	60	65	70	75
Ficin k , min^{-1}	0,015 $\pm 0,006$	0,056 $\pm 0,004$	0,185 $\pm 0,007$	0,56 $\pm 0,01$

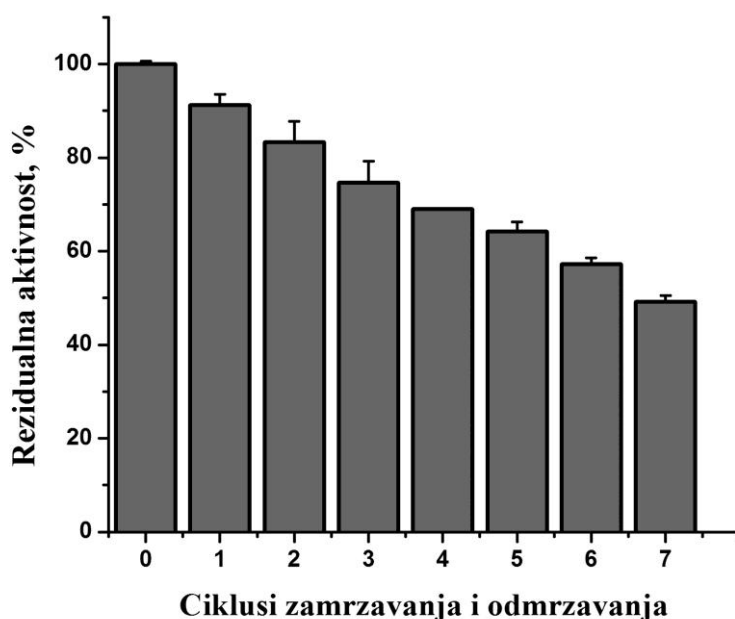
Detektovano je povećanje konstante brzine inaktivacije ficina više od 37 puta na 75 °C u odnosu na 60 °C (tabela 5.5).

Na osnovu Arenijusovog grafika (slika 5.16B) izračunata je vrednost aktivacione energije inaktivacije ficina koja je iznosila 233 ± 4 kJ/mol.

5.8.2. Ispitivanje stabilnosti ficina na niskim temperaturama

5.8.2.1. Ispitivanje aktivnosti ficina na niskim temperaturama

Aktivnost ficina tokom uzastopnih ciklusa zamrzavanja/odmrzavanja na -20 °C praćena je korišćenjem supstrata BAPNA. Merena je rezidualna aktivnost nakon svakog od 7 ciklusa zamrzavanja i odmrzavanja. Rezultati praćenja enzimske aktivnosti ficina prikazani su na slici 5.26.

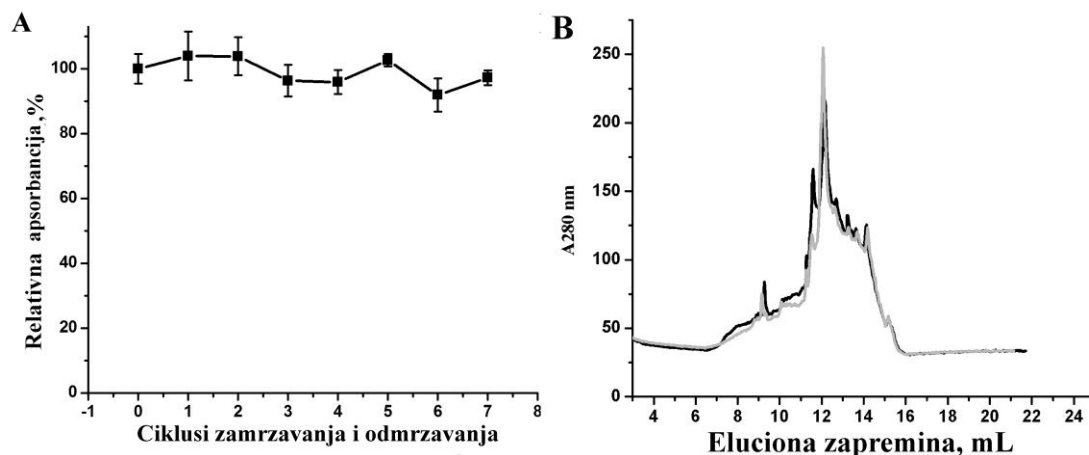


Slika 5.26. Aktivnost ficina tokom sedam ciklusa zamrzavanja/odmrzavanja. Aktivnost startnog uzorka ficina (0) posmatrana je kao 100%, dok su izmerene aktivnosti nakon svakog ciklusa (1-7) izražene kao procenti startne aktivnosti. Prikazani rezultati predstavljaju srednje vrednosti \pm standardne devijacije rezultata dobijenih iz tri nezavisna eksperimenta.

Iz dobijenih rezultata zapaža se da usled zamrzavanja/odmrzavanja dolazi do postepenog pada aktivnosti ficina. Nakon svakog od ciklusa ficin izgubi oko 5 do 8% aktivnosti. Kada se uporedi aktivnost nultog (netretiranog) uzorka ficina i uzorka ficina nakon 7. ciklusa zamrzavanja/odmrzavanja, preostaje oko 50% aktivnosti (slika 5.26).

5.8.2.2. Ispitivanje stabilnosti primarne strukture ficina na niskim temperaturama

Potencijalne promene u primarnoj strukturi ficina izazvane autoproteolizom praćene su nakon svakog ciklusa zamrzavanja/odmrzavanja i rezultat je prikazan na slici 5.27.



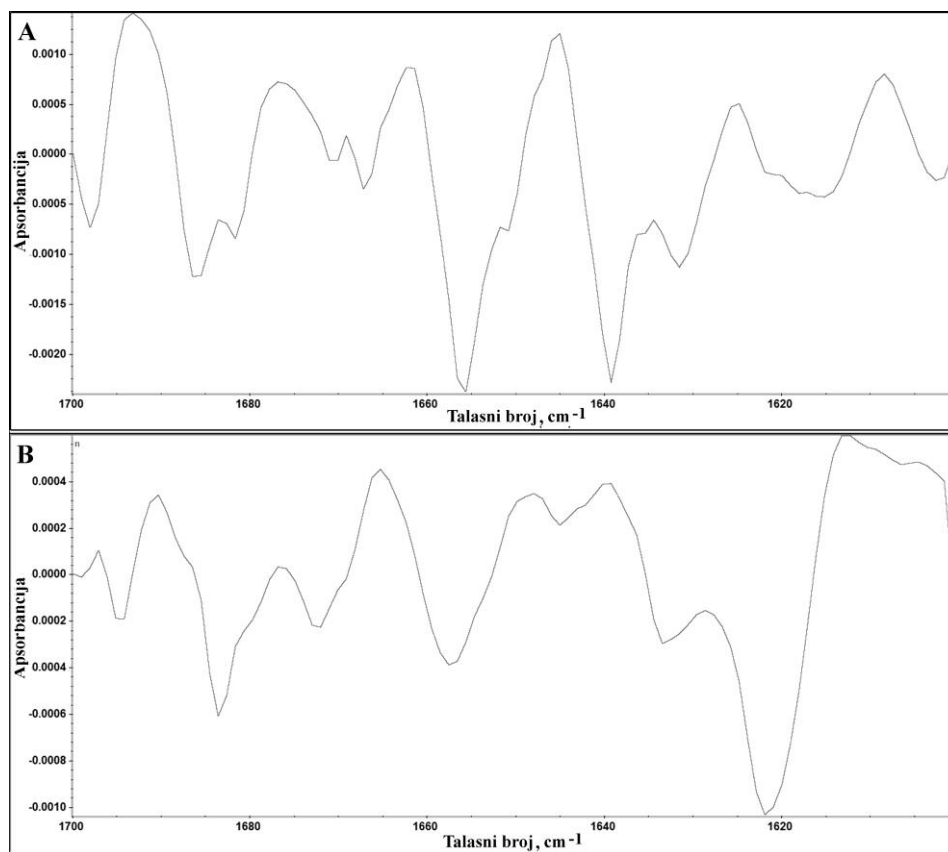
Slika 5.27. A) Autoproteoliza ficina tokom sedam ciklusa zamrzavanja/odmrzavanja. Apsorbancija nakon svakog ciklusa izražena je kao procenat inicijalne apsorbancije; B) RP-HPLC nativnog ficina (crna linija) i ficina čuvanog na niskoj temperaturi (siva linija).

Rezultat praćenja autoproteolize ficina pokazuje da se stepen vezivanja boje CBB za papain smanjuje najviše oko 10% (slika 5.27A), mada ne statistički značajno. S druge strane, nema značajne razlike ni u reverzno-faznim hromatogramima nativnog ficina i ficina izlaganog uzastopnim ciklusima zamrzavanja/odmrzavanja (slika 5.27B).

5.8.2.3. Ispitivanje sekundarnih struktura ficina na niskim temperaturama

Infracrveni spektri sa Furijeovom transformacijom snimljeni su za nativni ficin, kao i za ficin inkubiran na niskoj temperaturi (nakon 7 ciklusa zamrzavanja/odmrzavanja).

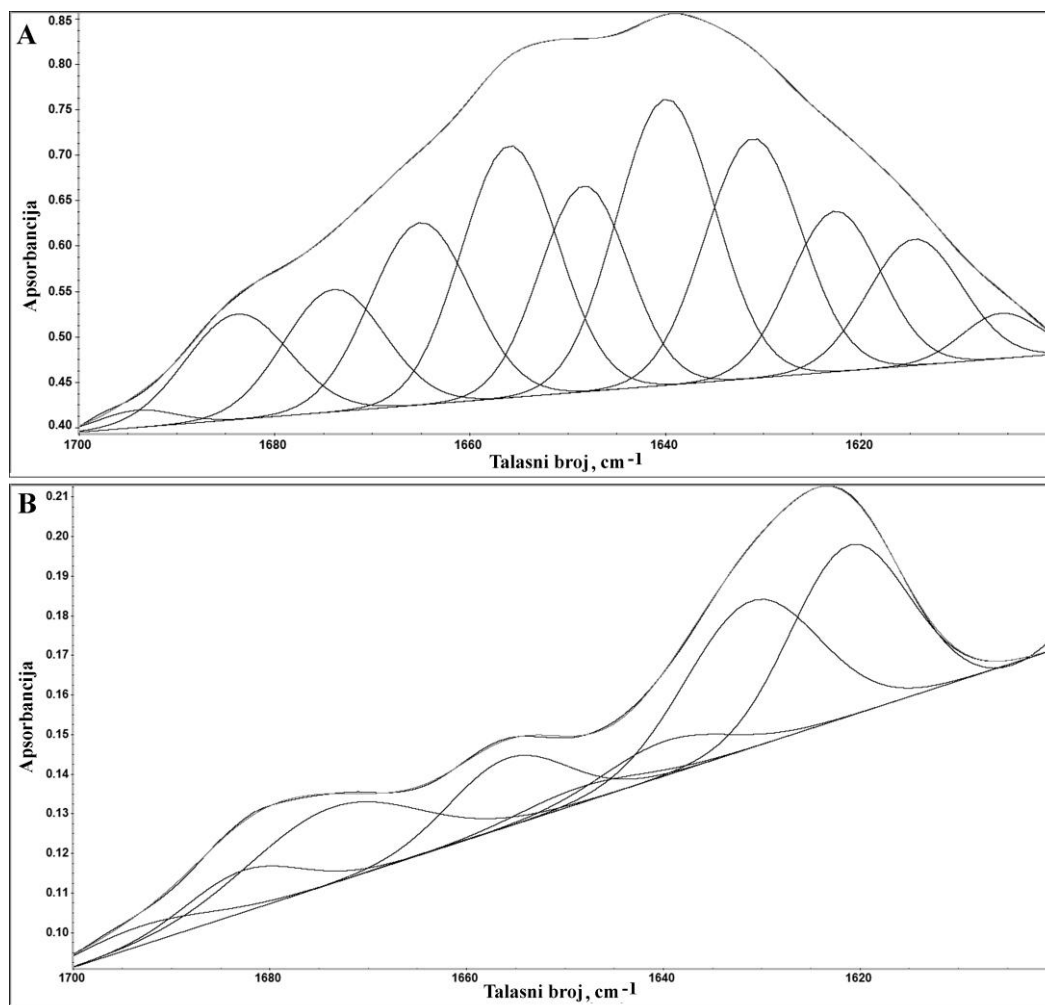
Poređenje drugih izvoda spektara amidnog I regiona prikazano je na slici 5.28.



Slika 5.28. Drugi izvodi FT-IR spektara: A) nativnog ficina; B) ficina inkubiranog na niskoj temperaturi (nakon 7 ciklusa zamrzavanja/odmrzavanja).

U drugom izvodu spektra nativnog ficina može se uočiti nekoliko pikova (minimума). Najintenzivnije trake uočavaju se naročito u regionu koji se dodeljuje uređenim sekundarnim strukturama i to na sledećim talasnim brojevima: 1631, 1640, 1649, 1656, 1664, 1666, 1672, 1680-1686 cm^{-1} , 1697 cm^{-1} kao i trake ispod 1620 cm^{-1} koje potiču od slobodne karboksilne grupe bočnih ostataka [145] (slika 5.28A). Najizraženija razlika u drugom izvodu spektra ficina koji je tretiran uzastopnim ciklusima zamrzavanja/odmrzavanja (slika 5.28B) jeste nestajanje trake na 1649 cm^{-1} , koja se pripisuje α -heliksu [135], kao i pojavljivanje intenzivne široke trake na 1622 cm^{-1} ukazujući na pojavu intermolekulske β -pločice [135, 136] (slika 5.28B).

Nakon razlaganja FT-IR spektara na komponente (slika 5.29), pikovi su dodeljeni specifičnim sekundarnim strukturama (tabela 5.6) i procenat svake sekundarne strukture izračunat je na osnovu površina ispod karakterističnih pikova u odnosu na površinu čitavog amidnog I regiona. Rezultati izračunavanja sadržaja sekundarnih struktura prikazani su u tabeli 5.7.



Slika 5.29. Originalni i fitovani FT-IR spektar: A) nativnog ficina; B) ficina inkubiranog na niskoj temperaturi (nakon 7 ciklusa zamrzavanja/odmrzavanja).

Pored informacija o položaju i širini karakterističnih traka koje su dobijene prebacivanjem originalnih spektara u drugi izvod, dodatne informacije o sadržaju različitih sekundarnih struktura u nativnom ficinu i ficinu nakon sedam ciklusa zamrzavanja/odmrzavanja dobijene su nakon razlaganja spektara na komponente (slika 5.29). Najznačajnija razlika je porast površine ispod krive koja se dodeljuje intermolekularnoj β -pločici (1622 cm^{-1}) usled uzastupnog zamrzavanja/odmrzavanja.

Tabela 5.6. Dodeljivanje traka amidnog I regiona karakterističnim sekundarnim strukturama i relativne površine ispod traka u odnosu na kompletni amidni I region u spektrima nativnog ficina i ficina nakon sedam uzastopnih ciklusa zamrzavanja/odmrzavanja. Relativne površine ispod traka (u procentima) određene su metodom fitovanja u krivu. Inter - intermolekulska β -pločica; intra - intramolekulska β -pločica.

Nativni ficin		Ficin nakon 7 ciklusa zamrzavanja/odmrzavanja		Dodeljena sekundarna struktura
ν (cm ⁻¹)	Površina (%)	ν (cm ⁻¹)	Površina (%)	
1622	9,6	1622	27,0	Inter/intra β -pločica [135, 136]
1631	15,3	1631	26,2	Intra β -pločica [135]
1640	18,6	1640	5,8	Neuređeni niz [135]
1649	12,1	1648	2,6	α -heliks [135]
1656	16,0	1656	10,9	α -heliks [135]
1666	12,1	1663	0,1	Zavijutak [56, 135, 136]
1672	8,1	1673	17,4	Neuređeni heliks [136]
1684	7,2	-	-	Zavijutak [135, 137]
-	-	1684	6,5	Zavijutak [135, 137]
1697	0,9	1693	3,5	Inter/intra β -pločica [135, 136]

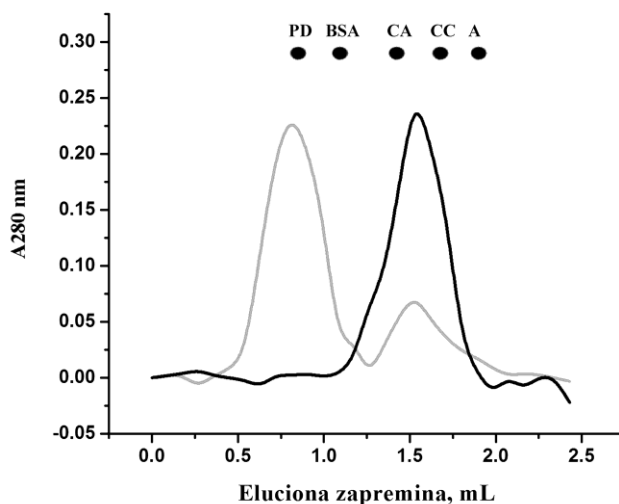
Tabela 5.7. Sadržaj sekundarnih struktura (%) dobijen fitovanjem u krivu prisutan u nativnom ficinu i ficinu inkubiranom na niskoj temperaturi (nakon sedam uzastopnih ciklusa zamrzavanja/odmrzavanja).

Uzorak ficina	Sadržaj sekundarnih struktura (%)					Greška
	β -pločica	α -heliks	Zavijutak	Neuređene strukture	Inter/Intra β -pločica	
Nativni ficin	15,3	28,2	19,3	26,7	10,5	2,3
Ficin inkubiran na niskoj temperaturi	26,2	13,5	6,6	23,2	30,5	0,4
CD [88]	22-26	19-24	18-19	35-40		

Rezultat upoređivanja sadržaja sekundarnih struktura netretiranog ficina i ficina nakon 7 uzastopnih ciklusa zamrzavanja/odmrzavanja sumiran je u tabeli 5.7. Kao što je i na osnovu spektara zapaženo, nakon tretmana niskom temperaturom, dolazi do veoma izraženog porasta sadržaja intermolekulske β -pločice, i to najviše na račun smanjenja sadržaja α -heliksa čiji sadržaj opada sa 28,2% na 13,5%.

5.8.2.4. Ispitivanje agregiranja ficina tokom izlaganja niskim temperaturama

Agregiranje ficina usled skladištenja na niskoj temperaturi, po analogiji sa papainom, praćeno je gel-filtracijom visokih performansi. Dobijeni hromatogram prikazan je na slici 5.30.



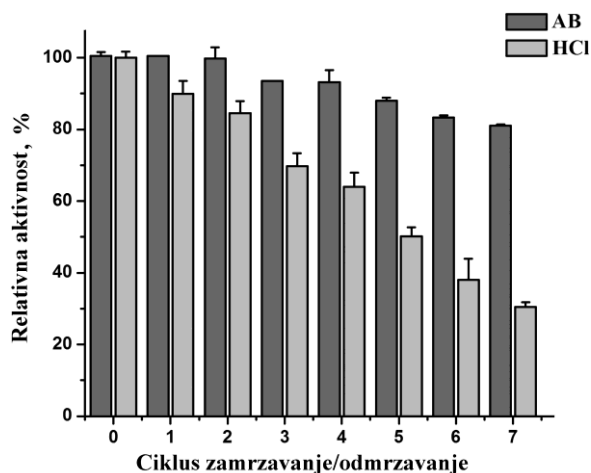
Slika 5.30. Gel-filtracija nativnog ficina (crna linija) i ficina inkubiranog na niskoj temperaturi (siva linija). PD, BSA, CA, CC i A - standardi molekulskih masa (PD - plavi dekstran 2000 kDa, BSA - goveđi serum albumin 67 kDa, CA - karbonska anhidraza 29 kDa, CC - citohrom c 14 kDa i A - aprotinin 6,5 kDa).

Na hromatogramu nativnog ficina zapaža se jedan dominantni pik koji odgovara izračunatoj masi od 24,0 kDa. Na hromatogramu ficina skladištenog na niskim temperaturama pik koji odgovara nativnom ficinu manjeg je intenziteta (17%), dok je ostatak ficina iz ovog uzorka eluiran u mrtvoj zapremini kolone (slika 5.30).

5.9. Ispitivanje stabilnosti tripsina na niskim temperaturama

5.9.1. Ispitivanje aktivnosti tripsina na niskim temperaturama

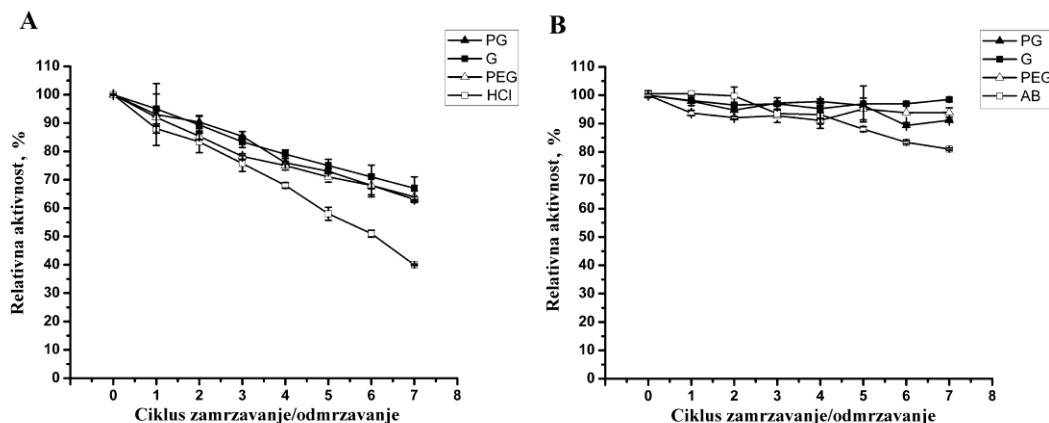
Ispitivane su promene aktivnosti tripsina (prema supstratu BAPNA) koje nastaju usled izlaganja tripsina niskoj temperaturi, odnosno, nakon svakog od 7 ciklusa zamrzavanja/odmrzavanja. Pritom je tripsin analiziran kako u kiselim uslovima (1 mM HCl po preporuci proizvođača), tako i u baznim uslovima (100 mM AB). Rezultati određivanja rezidualne aktivnosti nakon zamrzavanja/odmrzavanja prikazani su na slici 5.31.



Slika 5.31. Aktivnost tripsina tokom sedam ciklusa zamrzavanja/odmrzavanja. Aktivnost startnog uzorka tripsina (0) posmatrana je kao 100%, dok su izmerene aktivnosti nakon svakog ciklusa (1-7) izražene kao procenti startne aktivnosti. Rezultati predstavljaju srednje vrednosti tri nezavisna eksperimenta. AB - uzorak tripsina rastvoren u 100 mM amonijum-bikarbonatu; HCl - uzorak tripsina rastvoren u 1 mM hlorovodoničnoj kiselini.

Kada se tripsin rastvori u 1 mM hlorovodoničnoj kiselini, dolazi do linearnog gubitka aktivnosti usled zamrzavanja/odmrzavanja, pri čemu nakon sedmog ciklusa preostaje svega oko trećina početne aktivnosti (slika 5.31). Drugačija slika prisutna je ukoliko se tripsin podvrgne niskoj temperaturi u baznim uslovima, odnosno u 100 mM amonijum-bikarbonatu. Preko 80% aktivnosti biva, u tom slučaju, očuvano nakon 7 ciklusa zamrzavanja/odmrzavanja.

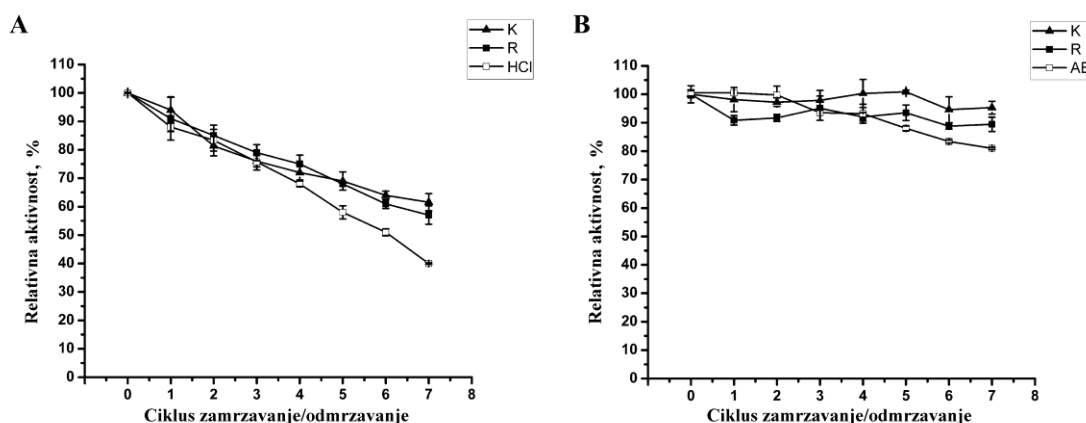
Ispitivan je i uticaj krioprotektanata na procenat očuvanja aktivnosti nakon zamrzavanja/odmrzavanja tripsina. Izabrani su poliolli (slika 5.32) i aminokiseline - lizin i arginin (slika 5.33). Lizin i arginin su odabrani jer je pretpostavljeno da ove aminokiseline akomodiranjem u aktivno mesto tripsina mogu da blokiraju/inhibiraju autoproteolizu, pošto je poznato da tripsin hidrolizuje peptidnu vezu iza ovih aminokiselinskih ostataka [91].



Slika 5.32. Aktivnost tripsina tokom sedam ciklusa zamrzavanja/odmrzavanja. Aktivnost startnog uzorka tripsina (0) posmatrana je kao 100% aktivnosti, dok su izmerene aktivnosti nakon svakog ciklusa (1-7) izražene kao procenti startne aktivnosti. Rezultati predstavljaju srednje vrednosti tri nezavisna eksperimenta; A) Tripsin u kiselim uslovima: HCl - tripsin rastvoren u 1 mM HCl; PG, G i PEG - tripsin rastvoren u 1 mM HCl sa dodatim krioprotektantima - propilen-glikol, glicerol i polietilen-glikol; B) Tripsin u baznim uslovima: AB - tripsin rastvoren u 100 mM amonijum-bikarbonatu; PG, G i PEG - tripsin rastvoren u 100 mM amonijum-bikarbonatu sa dodatim krioprotektantima - propilen-glikol, glicerol i polietilen-glikol.

Kada se u rastvor tripsina dodaju polioli, u oba slučaja dolazi do značajno veće očuvanosti aktivnosti tripsina (slika 5.32A i B). I u kiselim i u baznim uslovima glicerol se pokazao kao najmoćniji krioprotektant, sa nešto većim procentom rezidualne aktivnosti u odnosu na propilen-glikol i polietilen-glikol (99% aktivnosti je očuvano sa glicerolom, u odnosu na 96%, odnosno 95% aktivnosti koja ostaje očuvana dodatkom polietilen-glikola, odnosno propilen-glikola). Međutim, uprkos povećanju procenta rezidualne aktivnosti u prisustvu poliola, ne može se u potpunosti kompenzovati gubitak aktivnosti u kiselim uslovima. Naime, maksimalan procenat rezidualne aktivnosti koji se može dobiti u kiselim uslovima, postiže se dodatkom glicerola i iznosi oko 70% (slika 5.32A). S druge strane, u baznim uslovima u prisustvu glicerola, aktivnost tripsina je potpuno očuvana i nakon 7 ciklusa zamrzavanja/odmrzavanja (slika 5.32B).

Ispitan je i efekat dodatka lizina i arginina kao aditiva na aktivnost tripsina usled zamrzavanja/odmrzavanja i rezultati su prikazani na slici 5.33.

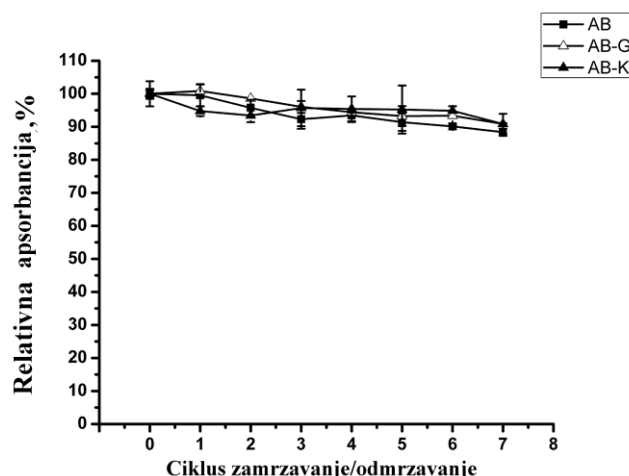


Slika 5.33. Aktivnost tripsina tokom sedam ciklusa zamrzavanja/odmrzavanja. Aktivnost startnog uzorka tripsina (0) posmatrana je kao 100% aktivnosti, dok su izmerene aktivnosti nakon svakog ciklusa (1-7) izražene kao procenti startne aktivnosti. Rezultati predstavljaju srednje vrednosti tri nezavisna eksperimenta; A) Tripsin u kiselim uslovima: HCl - tripsin rastvoren u 1 mM HCl; K, R - tripsin rastvoren u 1 mM HCl sa dodatim aditivima - lizin, arginin; B) Tripsin u baznim uslovima: AB - tripsin rastvoren u 100 mM amonijum-bikarbonatu; K, R - tripsin rastvoren u 100 mM amonijum-bikarbonatu sa dodatim aditivima - lizin, arginin.

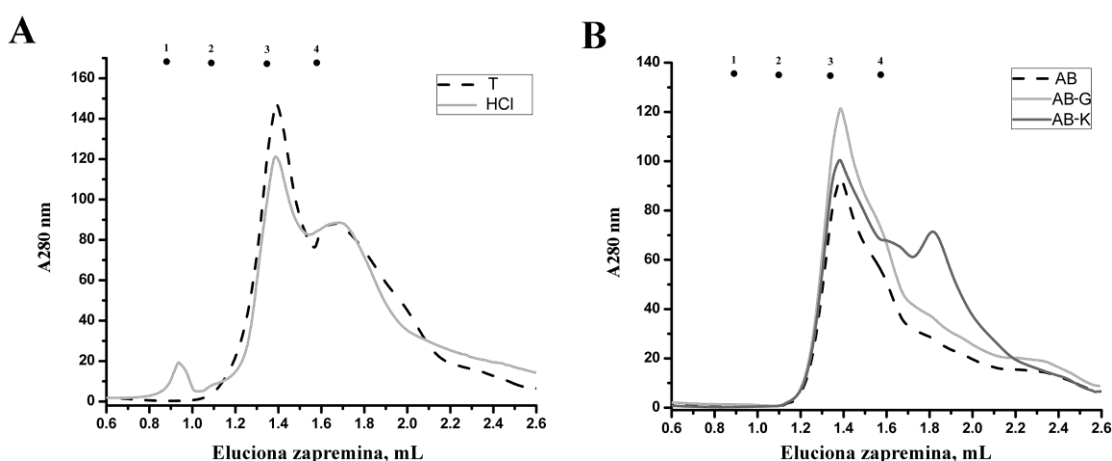
U prisustvu arginina i lizina zapaža se porast rezidualne aktivnosti tripsina nakon izlaganja niskoj temperaturi kako u kiselim, tako i u baznim uslovima (slika 5.33A i B). Međutim, maksimalan procenat rezidualne aktivnosti koji se dobija u kiselim uslovima je svega oko 60% (slika 5.33A), dok se u baznim uslovima postiže gotovo potpuno ($95 \pm 2\%$) očuvanje aktivnosti tripsina u prisustvu lizina i nešto manji procenat u prisustvu arginina ($89 \pm 2\%$) (slika 5.33B).

5.9.2. Ispitivanje stabilnosti primarne strukture tripsina na niskim temperaturama

Autoproteoliza tripsina tokom sedam ciklusa zamrzavanja/odmrzavanja praćena je testom vezivanja boje CBB. Rezultati su prikazani na slici 5.34. S druge strane, da bismo detektovali eventualne promene na primarnoj strukturi tripsina usled proteolize, rađena je i gel-filtracija visokih performansi (slika 5.35), kao i SDS-PAGE (prilog 6).



Slika 5.34. Autoproteoliza tripsina tokom sedam ciklusa zamrzavanja/odmrzavanja. Apsorbancija nakon svakog ciklusa izražena je kao procenat inicijalne apsorbancije.



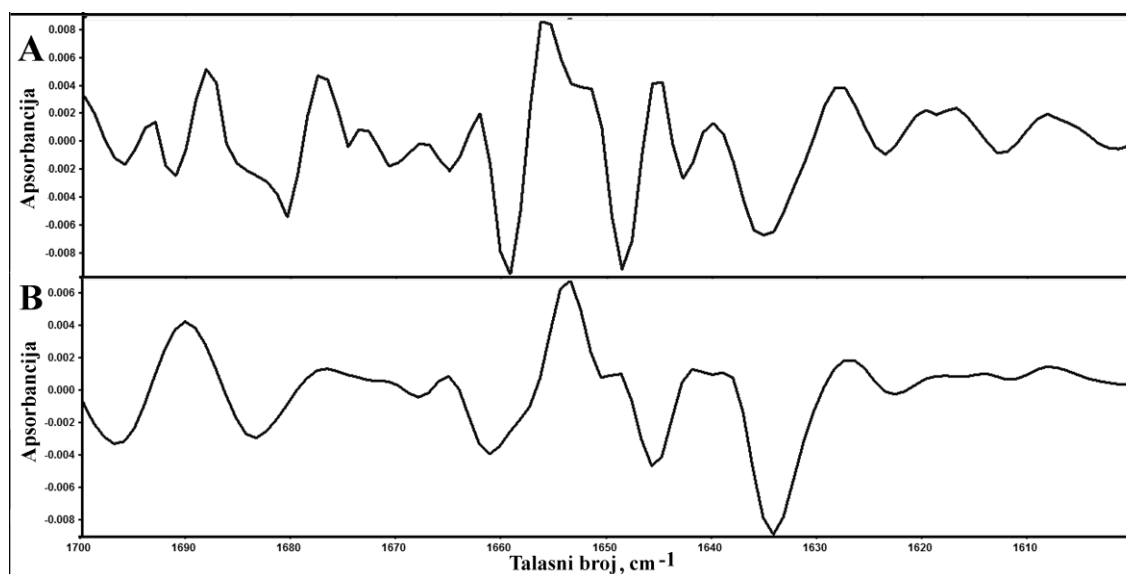
Slika 5.35. Gel-filtracija: A) nativnog tripsina rastvorenog u hlorovodoničnoj kiselini (HCl) i tripsina rastvorenog u hlorovodoničnoj kiselini nakon sedam ciklusa zamrzavanja/odmrzavanja (T); B) nativnog tripsina rastvorenog u amonijum-bikarbonatu (AB), amonijum-bikarbonatu i glicerolu (AB-G) i amonijum-bikarbonatu i lizinom (AB-K) nakon sedam ciklusa zamrzavanja/odmrzavanja. 1 - plavi dekstran, 2 - goveđi serum albumin, 3 - karbonska anhidraza, 4 - citohrom c.

Autoproteoliza nije detektovana u uzorcima tripsina rastvorenim u hlorovodoničnoj kiselini (rezultati nisu prikazani). Što se tiče uzoraka komercijalnog tripsina rastvorenih u amonijum-bikarbonatu (bez aditiva i sa glicerolom i lizinom), može se zapaziti da takođe nije detektovana značajna autoproteoliza nakon sedam ciklusa zamrzavanja/odmrzavanja u testu vezivanja CBB (manje od 10%) (slika 5.34).

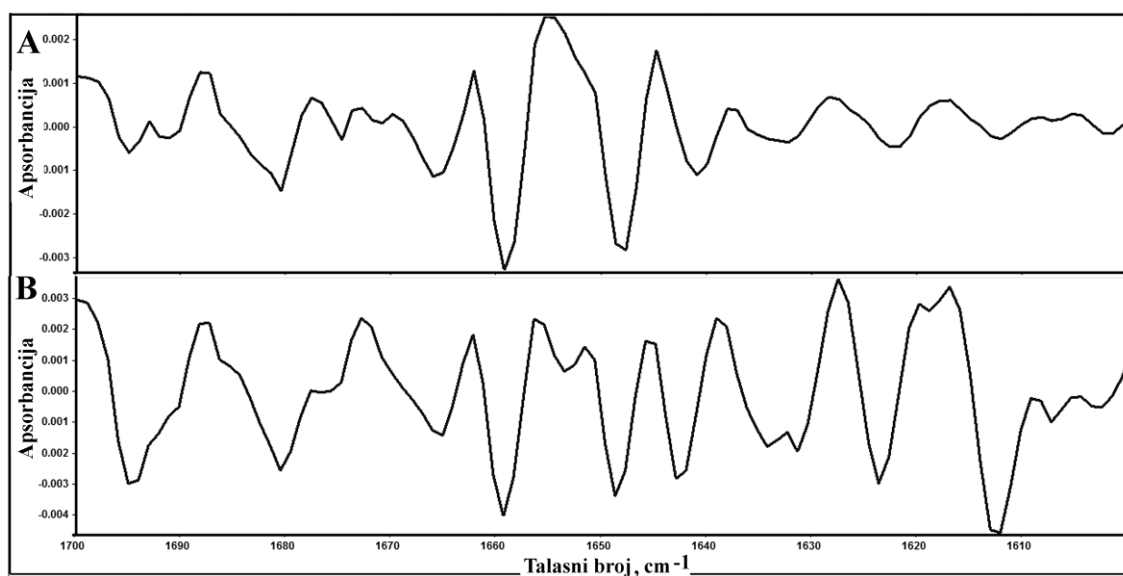
U gel-filtraciji detektovan je pik karakterističan za tripsin sa izračunatom molekulskom masom od 25 kDa u svim analiziranim uzorcima, a očuvanost ovog pika svedoči o veoma ograničenom nivou autoproteolize tripsina u alkalnoj sredini (slika 5.35). Pik koji odgovara proteinu manje molekulske mase detektovan je u netretiranom uzorku, kao i u zamrzavanom tripsinu u kiselim uslovima (slika 5.35A). Ovaj pik, međutim, nestaje tokom sedam ciklusa zamrzavanja/odmrzavanja u baznoj sredini, verovatno kao posledica proteolize, osim u prisustvu lizina (slika 5.35B).

5.9.3. Ispitivanje sekundarnih struktura tripsina na niskim temperaturama

Rezultati analize promena u sekundarnoj strukturi tripsina dobijeni su primenom metode infracrvene spektroskopije sa Furijeovom transformacijom. Drugi izvodi spektara prikazani su na slikama 5.36 i 5.37.



Slika 5.36. Drugi izvod amidnog I regiona FT-IR spektra sledećih uzoraka tripsina: A) Tripsin rastvoren u 1 mM hlorovodoničnoj kiselini; B) Tripsin rastvoren u 1 mM hlorovodoničnoj kiselini nakon sedam ciklusa zamrzavanja/odmrzavanja.



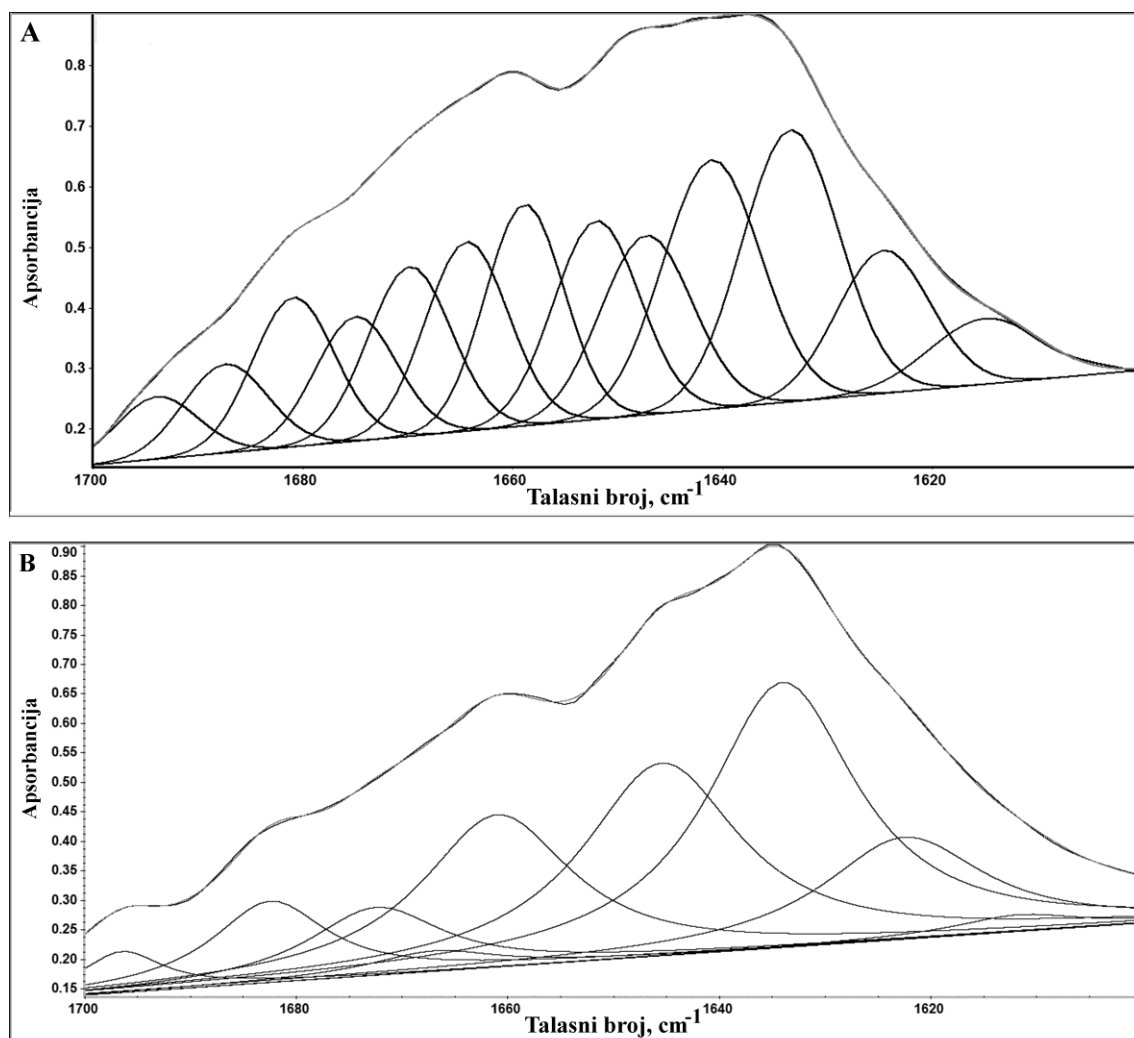
Slika 5.37. Drugi izvod amidnog I regiona FT-IR spektra sledećih uzoraka tripsina: A) Tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,3 M glicerolom (AB-G) nakon sedam ciklusa zamrzavanja/odmrzavanja; B) Tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,1 M lizinom (AB-K) nakon sedam ciklusa zamrzavanja/odmrzavanja.

Promene u amidnom I regionu IR spektra tripsina rastvorenog u 1 mM HCl uzrokovane ciklusima zamrzavanja/odmrzavanja prikazane su na slici 5.36. Slika 5.36A prikazuje drugi izvod spektra netretiranog tripsina rastvorenog u 1 mM hlorovodoničnoj kiselini. Glavne trake dodeljene β -pločicama detektovane su na talasnim brojevima 1625, 1634, 1675, 1681, 1688 [146] i iznad 1690 cm^{-1} [145, 147, 148]. Neuređeni niz prisutan je na položajima 1641 i 1648 cm^{-1} [146], dok pik pronađen na 1659 cm^{-1} pripada neuređenom heliksu [147]. Uređeni heliks uočava se samo u drugom izvodu spektra (1653 cm^{-1}) [56, 137, 147] (slika 5.36A). Spektar tripsina koji je podvrgnut ciklusima zamrzavanja/odmrzavanja (slika 5.36B) kao najdominantniju razliku pokazuje maksimum na 1634 cm^{-1} , ukazujući na povišeni sadržaj strukture β -pločice [146], kao i manje izražene pikove koji pripadaju neuređenim strukturama.

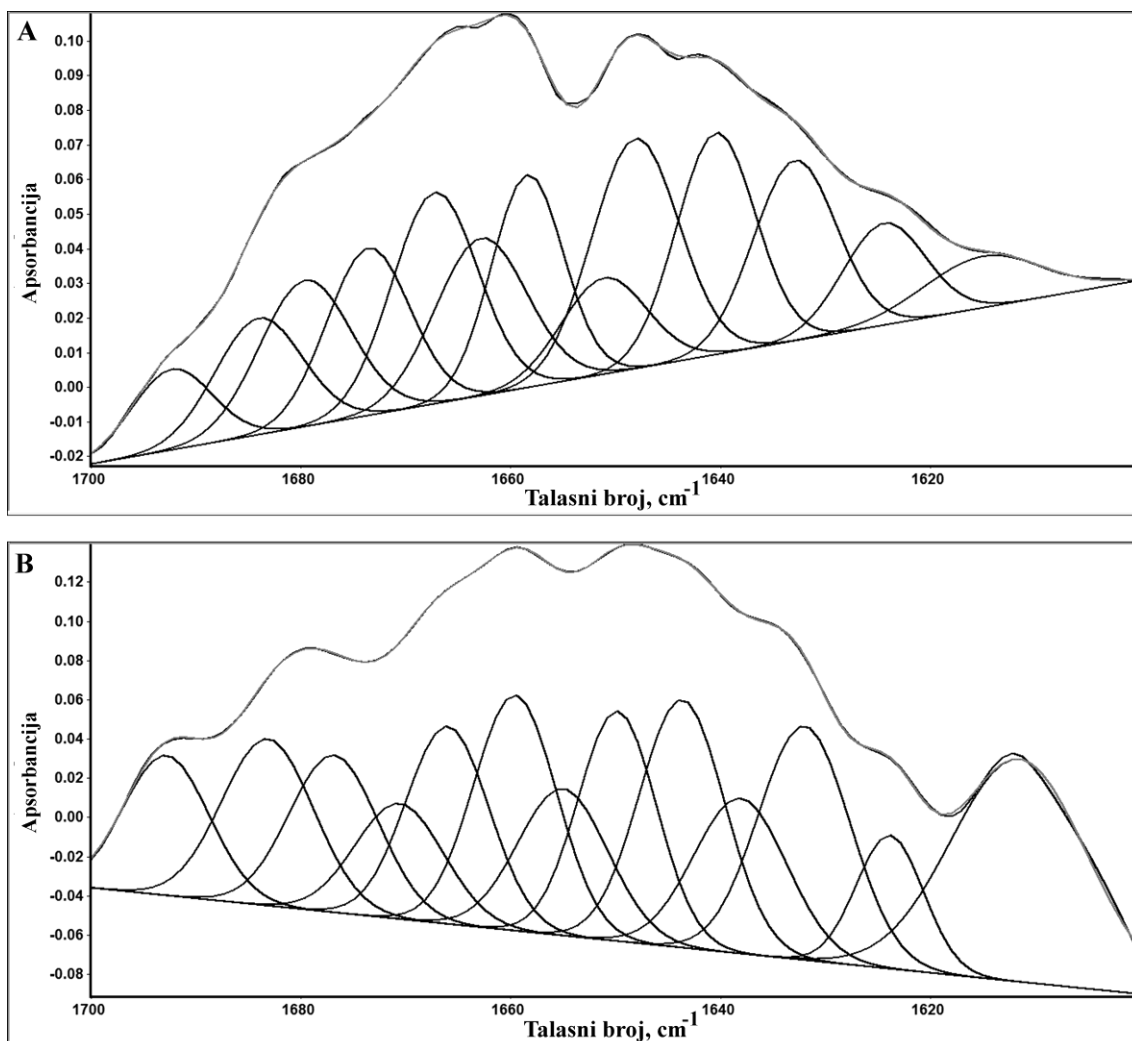
Drugi izvod spektra (amidni I region) uzoraka tripsina u baznim uslovima (100 mM amonijum-bikarbonat) u prisustvu 0,3 M glicerola (slika 5.37A), odnosno 0,1 M lizina (slika 5.37B) međusobno se ne razlikuju značajno kada se uzme u obzir položaj glavnih traka i njihovi intenziteti. Najdominantnije trake pripadaju neuređenim strukturama i β -pločicama. Međutim, čak i jednostavnim posmatranjem originalnog spektra tripsina u kiselim uslovima (slika 5.36A) i baznim (slika 5.37A i B), može se zaključiti da postoji

izraženiji pik na 1634 cm^{-1} čak i bez zamrzavanja. Porast ove trake je još izraženiji nakon sedam ciklusa zamrzavanja/odmrzavanja (slika 5.36B).

Izračunati su sadržaji sekundarnih struktura uzoraka tripsina dobijenih iz FT-IR spektara, kao i sadržaj koji odgovara tercijarnoj strukturi utvrđenoj metodom difrakcije X-zraka (iz tercijarne strukture) tripsina (UniProt ulaz P00760 (TRY1_BOVIN)). Rezultati ovog poređenja prikazani su u tabeli 5.8.



Slika 5.38. Originalni i fitovani FT-IR spektri sledećih uzoraka tripsina: A) Tripsin rastvoren u 1 mM hlorovodoničnoj kiselini; B) Tripsin rastvoren u 1 mM hlorovodoničnoj kiselini nakon sedam ciklusa zamrzavanja/odmrzavanja.



Slika 5.39. Originalni i fitovani FT-IR spektarsledećih uzoraka tripsina: A) Tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,3 M glicerolom (AB-G) nakon sedam ciklusa zamrzavanja/odmrzavanja; B) Tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,1 M lizinom (AB-K) nakon sedam ciklusa zamrzavanja/odmrzavanja.

Tabela 5.8. Dodeljivanje traka amidnog I regiona karakterističnim sekundarnim strukturama i relativne površine ispod traka u odnosu na kompletni amidni I region u spektrima sledećih uzoraka tripsina: tripsin rastvoren u 1 mM hlorovodoničnoj kiselini (netretirani), tripsin rastvoren u 1 mM hlorovodoničnoj kiselini nakon sedam ciklusa zamrzavanja/odmrzavanja (HCl), tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,3 M glicerolom (AB-G) nakon sedam ciklusa zamrzavanja/odmrzavanja i tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,1 M lizinom (AB-K) nakon sedam ciklusa zamrzavanja/odmrzavanja. Relativne površine ispod traka (u procentima) određene su metodom fitovanja u krivu.

Netretirani tripsin		HCl		AB-G		AB-K		Dodeljena sekundarna struktura
ν (cm ⁻¹)	Površina (%)	ν (cm ⁻¹)	Površina (%)	ν (cm ⁻¹)	Površina (%)	ν (cm ⁻¹)	Površina (%)	
1625	5,6	1621	8,4	1624	5,2	1624	5,3	Inter/intra β [135, 136]
1634	20,1	1634	38,3	1633	15,1	1633	13,6	β -pločica [146]
1641	11,0	1645	11,2	1640	10,9	1644	22,0	Neuređeni niz [146]
1648	9,5	-	-	1648	10,6	-	-	Neuređeni niz [146]
1653	6,5	1652	5,6	1651	8,2	1651	7,8	α -heliks [56, 137, 147]
1659	8,5	1661	11,3	1658	8,5	1659	9,8	Neuređeni niz [147]
1665	8,9	1667	4,5	1663	8,0	1666	14,1	Zavijutak [146]
1670	7,5	1683	9,4	1667	6,3	-	-	Zavijutak [146]
1675	6,7	1673	9,3	1674	8,6	-	-	β -pločica [146]
1681	8,4	-	-	1680	8,3	1680	21,0	β -pločica [146]
1688	4,8	-	-	1684	6,3	-	-	β -pločica [146]
1694	2,5	1696	2,0	1692	4,0	1693	6,4	Inter/intra β [135, 136]

Tabela 5.9. Sadržaj sekundarnih struktura (%) dobijen fitovanjem u krivu spektara dobijenih primenom ATR FT-IR spektroskopije, ali je sadržaj sekundarnih struktura izračunat i iz podataka dobijenih difrakcijom X-zraka. Uzorci: tripsin rastvoren u 1 mM HCl (netretirani) i uzorci tripsina nakon 7 ciklusa zamrzavanja/odmrzavanja: rastvoren u 100 mM amonijum-bikarbonatu uz dodatak 0,3 M glicerola (AB-G), odnosno 0,1 M lizina (AB-K).

Uzorak tripsina	Sadržaj sekundarnih struktura (%)					
	β -pločica	α -heliks	Zavijutak	Neuređeni niz	Inter/intra β	Greška
Netretirani tripsin u HCl	40,1	6,5	16,4	28,9	8,1	3,7
HCl	47,6	5,6	13,9	22,4	10,4	2,1
AB-G	38,3	8,2	14,3	30,0	9,2	3,2
AB-K	34,6	7,8	14,1	31,8	11,7	5,3
Difrakcija X-zraka	46,2	8,1	12,1	33,6		-

Sadržaj sekundarnih struktura određen FT-IR spektroskopijom uporediv je sa sadržajem izračunatim iz podataka kristalne strukture tripsina u slučaju netretiranog tripsina i tripsina zamrzavanog u baznim uslovima. Kod uzorka tripsina zamrzavanog u kiselim uslovima došlo je do značajnog porasta sadržaja β -pločica i smanjenja procenta α -heliksa i neuređenih struktura (tabela 5.9). Sam porast intermolekulske β -pločice u slučaju tripsina zamrzavanog u kiselim uslovima je svega 2,3%. Ipak, kada se uporede podaci o sekundarnim strukturama izračunati iz kristalne strukture tripsina, postoje male razlike između tripsina zamrzavanog u baznim uslovima i netretiranog tripsina rastvorenog u HCl. Izračunati korelacioni koeficijenti ukazuju da, iako se svaki uzorak tripsina koji je podvrgnut ciklusima zamrzavanja/odmrzavanja razlikuje od kontrole (korelacioni koeficijenti znatno manji od 1,0), spektar tripsina rastvorenog u 100 mM amonijum-bikarbonatu bio je najslbližnji netretiranom tripsinu, dok se tripsin zamrzavan u HCl veoma razlikovao u odnosu na netretirani (tabela 5.10).

Tabela 5.10. Korelacioni koeficijenti spektara netretiranog tripsina rastvorenog u 1 mM HCl i uzoraka tripsina nakon sedam ciklusa zamrzavanja/odmrzavanja: tripsina rastvorenog u 1 mM HCl (HCl); tripsina rastvorenog u 100 mM amonijum-bikarbonatu sa 0,3 M glicerolom (AB-G) i tripsina rastvorenog u 100 mM amonijum-bikarbonatu sa 0,1 M lizinom (AB-K).

Uzorak	r vrednost (koeficijent korelacije spektara)
HCl	0.5536
AB-G	0.7452
AB-K	0.7991

5.9.4. Praćenje aktivnosti tripsina u peptidnom mapiranju metodom otiska prsta

Za različito tretirane uzorke tripsina finalno je izveden i eksperiment praćenja aktivnosti i funkcionalnosti metode za koju se tripsin komercijalno primenjuje - peptidno mapiranje metodom otiska prsta. Kao supstrat korišćen je BSA, te su rezultati digestije BSA tripsinom, kao i identifikacija programom MASCOT prikazani u tabelama koje slede (prilog 7, tabela 5.11).

Tabela 5.11. Primena različito tretiranih uzoraka tripsina u metodi peptidnog mapiranja - otisak prsta. Uzorci tripsina: netretirani tripsin rastvoren u 1 mM HCl i uzorci tripsina nakon sedam ciklusa zamrzavanja/odmrzavanja: tripsin rastvoren u 1 mM HCl (HCl); tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,3 M glicerolom (AB-G) i tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,1 M lizinom (AB-K).

Uzorak tripsina	Rezultat programa Mascot	Vrednost očekivanja	Broj identifikovanih peptida	Pokrivenost strukture, %
Netretirani	161	$5.3e^{-12}$	34	45
HCl	53	0.33	16	31
AB-G	159	$8.3e^{-12}$	34	44
AB-K	157	$1.3e^{-11}$	33	51

Fragmenti koji potiču od autoproteolize tripsina takođe su detektovani u digestovanim uzorcima i rezultati su prikazani u prilogu 8. U svim digestovanim uzorcima detektovano je dvanaest peptida, osim u tripsinu koji je zamrzavan i odmrzavan u prisustvu 0,1 M lizina (9 peptida).

5.9.5. Određivanje aktivacione energije inaktivacije tripsina

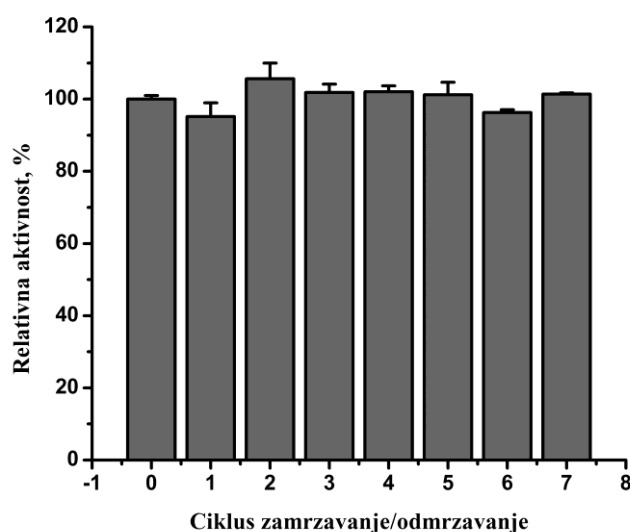
Aktivacione energije inaktivacije određene su za uzorke tripsina zamrzavane u kiselim i baznim uslovima (amonijum-bikarbonat) po ciklusu zamrzavanja/odmrzavanja. Za tripsin u amonijum-bikarbonatu sa glicerolom, odnosno lizinom nije bilo moguće izračunati iste s obzirom na nedovoljne razlike u aktivnostima nakon pojedinačnih ciklusa zamrzavanja/odmrzavanja (razlike nisu bile statistički značajne).

Dobijena vrednost aktivacione energije inaktivacije tripsina u kiselim uslovima iznosila je $21,4 \pm 0,2$ kJ/mol po ciklusu, dok je za tripsin u amonijum-bikarbonatu iznosila $24,6 \pm 0,2$ kJ/mol po ciklusu zamrzavanja/odmrzavanja.

5.10. Ispitivanje stabilnosti kolagenaze na niskim temperaturama

5.10.1. Ispitivanje aktivnosti kolagenaze na niskim temperaturama

Aktivnost kolagenaze tokom izlaganja uzastopnim ciklusima inkubiranja na -20 °C praćena je zimografski, korišćenjem želatina kao supstrata. Denzitometrijski je merena rezidualna aktivnost nakon svakog od 7 ciklusa zamrzavanja i odmrzavanja. Rezultati praćenja enzimske aktivnosti kolagenaze prikazani su na slici 5.40.

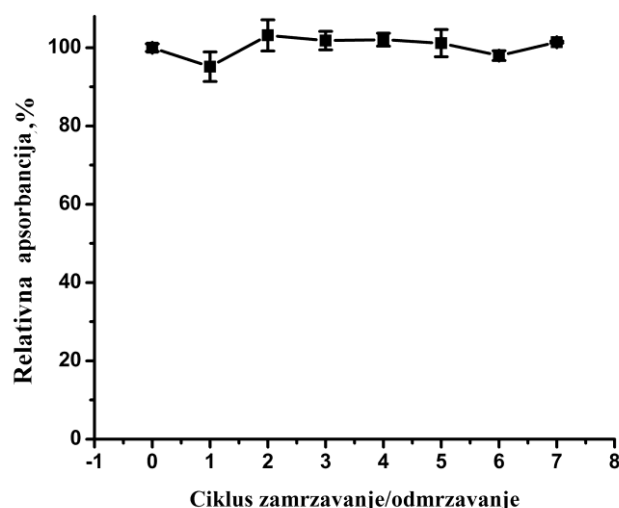


Slika 5.40. Aktivnost kolagenaze tokom sedam ciklusa zamrzavanja/odmrzavanja. Aktivnost početnog uzorka kolagenaze (0) posmatrana je kao 100%, dok su izmerene aktivnosti nakon svakog ciklusa (1-7) izražene kao procenti početne aktivnosti. Prikazani rezultati predstavljaju srednje vrednosti \pm standardne devijacije rezultata dobijenih iz tri nezavisna eksperimenta.

Aktivnost kolagenaze ostaje gotovo u potpunosti očuvana (u granicama greške) čak i nakon svih sedam ciklusa zamrzavanja/odmrzavanja (slika 5.40).

5.10.2. Ispitivanje stabilnosti primarne strukture kolagenaze na niskim temperaturama

Promene u primarnoj strukturi kolagenaze izazvane eventualnom autoproteolizom praćene su nakon svakog ciklusa zamrzavanja/odmrzavanja i rezultat je prikazan na slici 5.41.

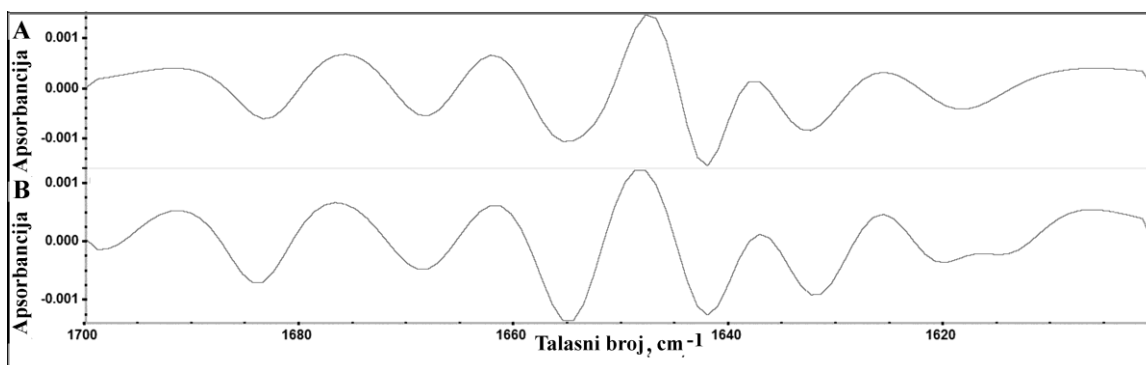


Slika 5.41. Autoproteoliza kolagenaze tokom sedam ciklusa zamrzavanja/odmrzavanja. Apsorbancija nakon svakog ciklusa izražena je kao procenat inicijalne apsorbancije.

Rezultat praćenja autoproteolize kolagenaze pokazuje da nema značajne autoproteolitičke aktivnosti tokom sedam ciklusa zamrzavanja/odmrzavanja. Step en vezivanja boje CBB pada manje od 10% (slika 5.41).

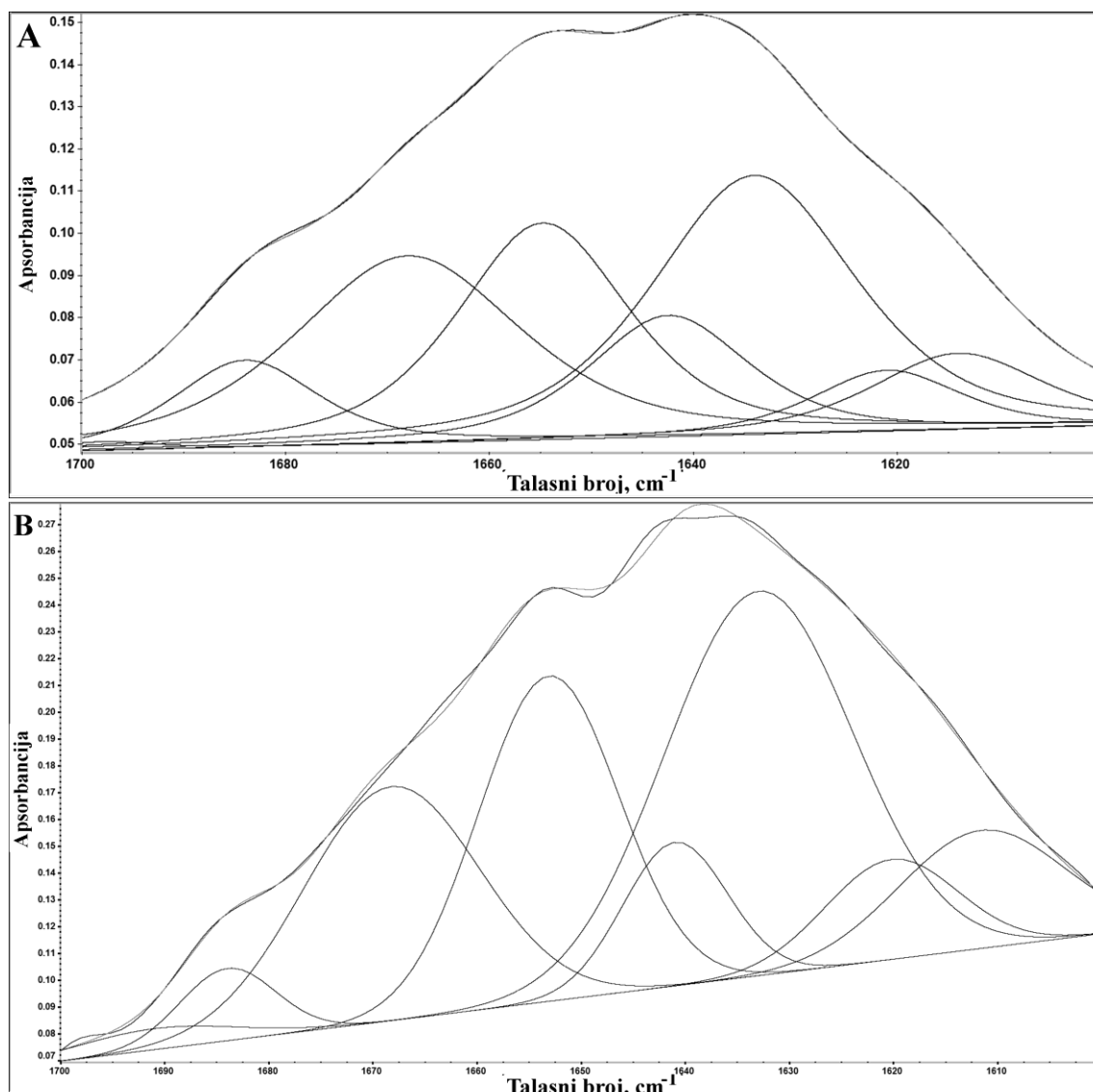
5.10.3. Ispitivanje sekundarnih struktura kolagenaze na niskim temperaturama

Infracrveni spektri sa Furijeovom transformacijom snimljeni su za nativnu formu kolagenaze, kao i za kolagenazu inkubiranu na niskoj temperaturi (nakon 7 ciklusa zamrzavanja/odmrzavanja). Promene u drugom izvodu spektara amidnog I regiona prikazane su na slici 5.42.



Slika 5.42. Drugi izvodi FT-IR spektara: A) nativne kolagenaze; B) kolagenaze inkubirane na niskoj temperaturi (nakon 7 ciklusa zamrzavanja/odmrzavanja).

Drugi izvod spektra native kolagenaze pokazuje da su pikovi prisutni na talasnim brojevima 1634, 1642, 1655 i 1684 cm^{-1} (slika 5.42A). Što se tiče pikova u spektru kolagenaze izlagane niskoj temperaturi, identifikovani su pikovi na istim pozicijama i sličnim intenzitetima kao i u spektru native kolagenaze (slika 5.42B).



Slika 5.43. Originalni i fitovani FT-IR spektar: A) native kolagenaze; B) kolagenaze inkubirane na niskoj temperaturi (nakon 7 ciklusa zamrzavanja/odmrzavanja).

Razlaganje originalnih spektara na komponente (slika 5.43) i dodeljivanje komponenata određenim sekundarnim strukturama (tabela 5.12) ukazuje da ne postoji značajna razlika ni u zastupljenosti, ni u položaju glavnih komponenti. I u sadržaju sekundarnih

struktura nije zapažena statistički značajna razlika posle 7 ciklusa zamrzavanja/odmrzavanja (tabela 5.13).

Tabela 5.12. Dodeljivanje traka amidnog I regiona karakterističnim sekundarnim strukturama i relativne površine ispod traka u odnosu na kompletni amidni I region u spektrima native kolagenaze i kolagenaze nakon sedam uzastopnih ciklusa zamrzavanja/odmrzavanja. Relativne površine ispod traka (u procentima) određene su metodom fitovanja u krivu. Inter - intermolekulska β -pločica; intra - intramolekulska β -pločica.

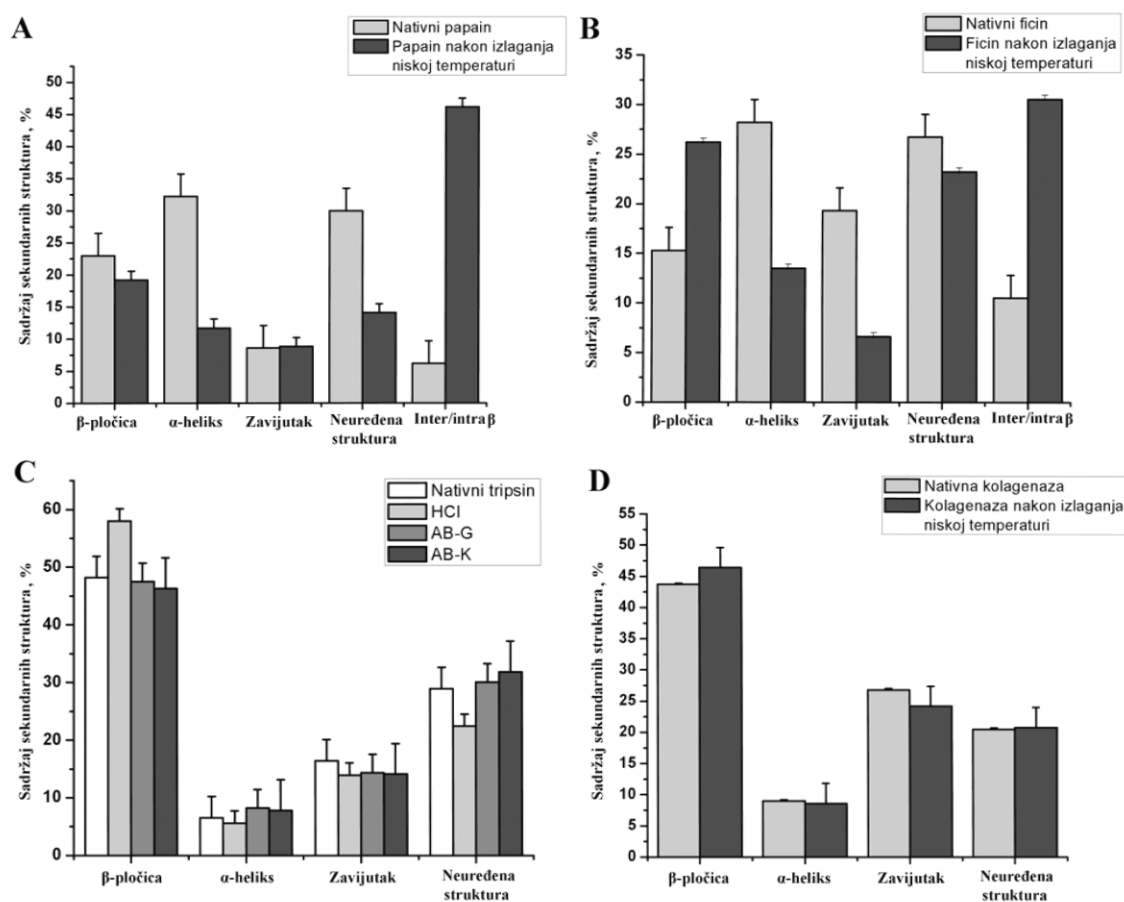
Nativna kolagenaza		Kolagenaza nakon 7 ciklusa zamrzavanja/odmrzavanja		Dodeljena sekundarna struktura
ν (cm^{-1})	Površina (%)	ν (cm^{-1})	Površina (%)	
1621	6,1	1621	6,7	Inter/intra β -pločica [137, 145]
1634	29,2	1633	34,5	β -pločica [135, 136]
1642	9,0	1641	8,6	Neuređeni niz [135]
1655	26,8	1654	24,2	α -heliks [56, 135-137]
1669	20,5	1668	20,8	Zavijutak [56, 135, 136]
1684	7,6	1684	3,5	β -pločica [135, 136]
1698	0,8	1698	1,7	Inter/intra β -pločica [56, 135, 149]

Tabela 5.13. Sadržaj sekundarnih struktura (%) dobijen fitovanjem u krivu prisutan u nativnoj kolagenazi i kolagenazi inkubiranoj na niskoj temperaturi (nakon sedam uzastopnih ciklusa zamrzavanja/odmrzavanja).

Uzorak kolagenaze	Sadržaj sekundarnih struktura (%)					
	β -pločica	α -heliks	Zavijutak	Neuređene strukture	Inter/Intra β -pločica	Greška
Nativna kolagenaza	36,8	26,8	20,5	9,0	6,9	0,2
Kolagenaza na niskoj temperaturi	38,0	24,2	20,8	8,6	8,4	3,2

5.11. Upoređivanje obrazaca strukturnih rearanžmana cistein- i serin-proteaza izazvanih izlaganjem niskim temperaturama

Histogramski prikaz (slika 5.44) pokazuje trendove strukturnih promena izazvanih niskom temperaturom, odnosno zamrzavanjem/odmrzavanjem kod svih ispitivanih model proteaza.



Slika 5.44. Sadržaj sekundarnih struktura (%) određenih primenom FT-IR spektroskopije sledećih proteaza: A) papain, B) ficin, C) tripsin, D) kolagenaza.

Kod cistein-proteaza, papaina i ficina, primećuje se sličan trend strukturnih promena; najizraženiji je porast procenta agregacionih β-pločica (za 40% kod papaina i za 20% kod ficina); u oba slučaja je zapažen i pad procenta α-heliksa (za 20,5% kod papaina i za 14,7% kod ficina) i neuređenih struktura (za 15,3% kod papaina i za 3,5% kod ficina) (slika 5.44A i 5.44B). Kod serin-proteaza ne detektuje se porast agregacionih β-pločica; čak se u slučaju kolagenaze ne detektuju ni promene u ostalim sekundarnim strukturama (slika 5.44D). Ni u slučaju tripsina koji je izlagan niskoj temperaturi u optimalnim uslovima (amonijum-bikarbonat pH 8) nisu detektovane statistički značajne promene (slika 5.44C). Nasuprot tome, u tripsinu koji je izlagan niskoj temperaturi u kombinaciji sa kiselim uslovima detektovane su sledeće promene: porast β-pločice za 7,5% i pad neuređenih struktura za 6,5%, kao i blagi pad procenta α-heliksa (slika 5.44C).

6. DISKUSIJA

U ovom istraživanju pokazano je da lateks smokve (*Ficus carica*) pored različitih izoformi cistein-proteaza, ficina, sadrži i novootkriveni enzim sa želatinolitičkom aktivnošću (slika 5.1). Naime, elektroforetski profil lateksa otkrio je do sada literaturno neopisanu proteinsku traku mase od oko 48 kDa, za razliku od mase ficina koje iznose oko 25 kDa (slika 5.1). I mapiranje po klasama proteaza lateksa nesumnjivo je pokazalo da je reč o novom proteinu, koji se razlikuje od do sada opisanih cistein-proteaza, ficina, pošto je pokazano da novootkriveni enzim pripada klasi serin-proteaza (slika 5.3). Mapiranje po klasama proteaza lateksa dalo je rezultat u kome su želatinolitička i kolagenolitička aktivnost bile potpuno inhibirane specifičnim inhibitorom serin-proteaza (PMSF), dok inhibitori specifični za ostale klase proteaza nisu imali uticaja na ove aktivnosti (slika 5.3). Kazeinolitička aktivnost lateksa, kao i aktivnost prema sintetičkom supstratu BAPNA, bile su gotovo potpuno inhibirane ireverzibilnim inhibitorima cistein-proteaza (IAA i E-64) i delimično inhibirane PMSF što se može objasniti pokazanom karakteristikom PMSF da reverzibilno inhibira i cistein-proteaze [150]. Opisani rezultati potvrđuju da su aktivnosti prema kazeinu i BAPNA supstratu nužno vezane za ficinske forme. Zanimljivo je da je u prisustvu EDTA, koji ima helirajuća svojstva te predstavlja specifični inhibitor metaloproteaza, aktivnosti i ficina i serin-proteaze blago porasle (slika 5.3). Ovaj porast aktivnosti može se objasniti eliminacijom malih količina teških metala koji su kao nečistoće prisutni u rastvorima, a koji inače mogu da inaktiviraju enzime, kao na primer, narušavanjem strukture/denaturacijom usled kompleksiranja sulfhidrilnih grupa [74].

Polazeći od literaturno poznatih karakteristika ficina, kao i razlika koje su pronađene između novootkrivene proteaze i ficina, optimizovana je procedura prečišćavanja koja se sastoji iz tri hromatografske metode i omogućava istovremeno izolovanje i ficina i kolagenaze (slike 5.4 i 5.5). Na ovaj način dobijeno je dobro razdvajanje (visok stepen prečišćenosti) ficina i kolagenaze (slika 5.6), dva model sistema ispitivanja stabilnosti proteaza na niskim temperaturama.

Novootkrivena serin-proteaza lateksa bazično je okarakterisana kao monomerni protein molekulske mase oko 41 kDa (slika 5.7) i pI vrednosti oko 5 (slika 5.8) koji pokazuje

stabilnost u širokom opsegu temperatura i pH vrednosti (prilog 1). Dok se ficinskim formama pripisuje široka supstratna specifičnost [63, 88, 151], koja je pokazana i u eksperimentu mapiranja proteaza lateksa po klasama (slika 5.3), za kolagenazu je pokazano da specifično hidrolizuje želatin (slika 5.1-5.3) i nativni kolagen (slika 5.9 i 5.10).

Kako mnogi enzimi široke supstratne specifičnosti mogu da hidrolizuju želatin i globularne terminalne regione kolagenskih lanaca, nakon bazične karakterizacije, cilj je bilo ispitivanje da li novootkriveni enzim može da hidrolizuje nativni kolagen u regionu trostrukog heliksa. Proizvodi digestije kolagenskih lanaca na SDS-PAGE ukazivali su na sposobnost hidrolize kako dimera (β lanci) i trimera kolagena (γ lanci), tako i monomernih $\alpha 1$ i $\alpha 2$ lanaca kolagena tipa I (slika 5.9). Finalno, primenom FT-IR spektroskopije bilo je moguće praćenje nestajanja trake karakteristične za trostruki heliks kolagena tokom inkubiranja sa prečišćenom serin-proteazom lateksa. Dobijeni FT-IR spektar nativnog kolagena sa karakterističnim trakama u sva tri amidna regiona (I-III) (slika 5.10) u saglasnosti je sa literaturnim podacima [152]. Pad intenziteta karakterističnih traka nakon inkubiranja sa kolagenolitičkom serin-proteazom pokazuje da dolazi do narušavanja strukture trostrukog heliksa kolagena. Naime, odnos karakterističnih traka infracrvenog spektra kolagena, odnos $1234\text{ cm}^{-1}/1450\text{ cm}^{-1}$, ukazuje na hidrolizu trostrukog heliksa nativnog kolagena, upravo zato što apsorpcija na talasnom broju 1234 cm^{-1} reflektuje očuvanost trake karakteristične za trostruki heliks u amidnom III regionu, dok je apsorpcija na 1450 cm^{-1} karakteristična za pirolidinski prsten [152]. Svi navedeni rezultati nedvosmisleno ukazuju na činjenicu da se serin-proteaza lateksa smokve može smatrati kolagenazom.

Kako kolagenaze nalaze široku primenu u medicini i biotehnologiji, postoji potreba za otkrivanjem novih kolagenaza [110, 111, 114, 115], naročito kada se uzme u obzir da je jedina komercijalno dostupna kolagenaza metalokolagenaza iz bakterijskog soja *Clostridium histolyticum* [153]. Ova komercijalna kolagenaza ima nedostatke (kao što je inaktiviranje u prisustvu helirajućih agenasa [59], ali i nepredvidiva i široka supstratna specifičnost koja je posledica kontaminacije drugim litičkim enzimima prisutnim u različitim preparatima kolagenaze koja i sama predstavlja smešu većeg broja izoformi [153]). Upravo iz priloženih razloga, novootkrivena serin-kolagenaza biljnog porekla

dobija na još većem značaju i ima potencijala za primenu u različitim granama biotehnologije i medicine.

Za ficin, kao model sistem ispitivanja stabilnosti na niskim temperaturama, nakon izolovanja potvrđeno je da predstavlja smešu većeg broja (izo)formi (slika 5.11), kao i komercijalni ficin opisan u prethodno publikovanoj literaturi [85, 88].

Papain, kao sledeći u nizu model sistema ove studije, prečišćen je iz komercijalnog preparata papaina do elektroforetske homogenosti (slika 5.12) u cilju uklanjanja neaktivnog/nenativnog papaina, kao i prisutnih pigmenata koji bi mogli da interferiraju sa korišćenim spektroskopskim metodama (prilog 2 i 3) i potom identifikovan TMF metodom (prilog 4).

Nakon izolovanja enzima koji su predstavljali model sisteme ove studije (uz bazičnu karakterizaciju kolagenaze), cilj je bilo ispitivanje stabilnosti cistein-proteaza (papaina i ficina) i serin-proteaza (tripsina za sekvenciranje i kolagenaze lateksa smokve) na niskim temperaturama.

Osim stabilnosti na visokim temperaturama, često je veoma važno ispitati ponašanje odnosno stabilnost komercijalnih enzima na niskim temperaturama, s obzirom da se neretko javlja potreba za skladištenjem ili primenom industrijskih enzima u procesima za koje je neophodna niska temperatura.

U cilju nalaženja optimalnog metodološkog pristupa za praćenje stabilnosti proteaza na niskim temperaturama, najpre je ispitana termalna stabilnost papaina (kao proteina koji je detaljno strukturno okarakterisan u literaturi) u širokom temperaturnom opsegu. Takođe, papain je okarakterisan i u kontekstu stabilnosti na visokim temperaturama, te je izabran i zbog mogućnosti poređenja sa već postojećom literaturom.

Diferencijalna UV spektroskopija predstavlja metodu koja se često koristi za praćenje denaturacije proteina. U UV regionu od 230 nm do 300 nm apsorbuju aromatične aminokiseline, ali njihov ekstinkcioni koeficijent u mnogome zavisi od njihovog okruženja. Okruženje diktira konformacija proteina, tako da su u nativnom molekulu aromatične aminokiseline obično unutar hidrofobnog jezgra proteina. Usled denaturacije, ekstinkcioni koeficijent ovih aminokiselinskih ostataka posledično se

menja čime utiče na spektar čitavog proteina [154-156]. Primenjena je, stoga, diferencijalna UV spektroskopija za praćenje stabilnosti papaina u širokom temperaturnom opsegu (od 5 do 99 °C) (slika 5.13). Međutim, ograničenje ove metode ogleda se u nemogućnosti praćenja denaturacije na temperaturama nižim od 5 °C zbog stvaranja leda u vodenom rastvoru proteina. UV spektroskopija na nižim temperaturama omogućila je detektovanje pada u procentu native strukture papaina, odnosno beleženje početka denaturacije na niskim temperaturama, pošto je na 5 °C nativna struktura bila redukovana za 18% (slika 5.14). Validnost praćenja termalne denaturacije i dobijenih rezultata diferencijalnom UV spektroskopijom, pokazana je određivanjem T_m vrednosti denaturacije papaina na povišenoj temperaturi, odnosno u opsegu u kom je detektovan potpuni prelaz native strukture u denaturisanu (od 100 do 0% native strukture) (slika 5.14). Dobijeni rezultat (84 ± 1 °C) u saglasnosti je sa prethodno publikovanom vrednošću od 83 ± 1 °C određenom diferencijalnom skenirajućom kalorimetrijom [3]. S druge strane, stabilnost native konformacije papaina u ovoj studiji praćena je i DSC metodom takođe u širokom temperaturnom intervalu (od 15 do 90 °C) (slika 5.14B). Time je utvrđeno da je UV spektroskopija dala T_m vrednost koja je u granicama greške identična vrednosti od 83,95 °C dobijenoj DSC metodom. Usled nemogućnosti praćenja promena strukture blizu i ispod tačke mržnjenja vode, u cilju određivanja T_c , istraživači standardno pribegavaju ekstrapoliranju termodinamičkih parametara snimljene krive koji karakterišu prelaze na visokim temperaturama (ukoliko je prisutan barem početak sigmoidnog prelaza native strukture na niskim temperaturama) [50, 157, 158]. Ekstrapoliranjem dobijenih vrednosti sve do 0% native strukture (slika 5.14A) izračunata je temperatura prelaza (na kojoj je 50% strukture nativno) u oblasti niskih temperatura (T_c vrednost) koja je iznosila -7 ± 1 °C za papain.

Metoda diferencijalne skenirajuće kalorimetrije imala je slična ograničenja kao i spektroskopske metode kada je određivanje stabilnosti na niskoj temperaturi u pitanju. Naime, ni ovom metodom nije bilo moguće doći do eksperimentalnih podataka o denaturaciji na niskim temperaturama. Takođe je zabeležen početak denaturacije na niskim temperaturama o čemu svedoči pad protoka toplote na temperaturama nižim od 40 °C (slika 5.14B).

Osim već navedenih karakteristika koje su papain postavile na prvo mesto ove studije, poznato je i da se papain smatra kinetički stabilnim enzimom [143]. Kinetički stabilni proteini pokazuju izraženu inertnost prema denaturaciji usled postojanja visoke barijere aktivacione energije između prelaznog stanja i native strukture, što je naročito karakteristično pri denaturaciji detergentom (SDS). Sledeći cilj ove studije bilo je optimizovanje metoda za praćenje strukturnih promena papaina na primeru ispitivanja uticaja SDS-a na strukturu papaina na povišenim temperaturama. Papain je u ovom kontekstu naročito pogodan model sistem upravo zbog postojanja dva domena od kojih se jedan dominantno sastoji od β -pločica, a drugi od α -heliksa. Postavljena je hipoteza da bi različiti domeni/različite sekundarne strukture pokazivale drugačiju podložnost termalnoj denaturaciji u prisustvu SDS-a, odnosno da bi bili različito kinetički inertni. Uzevši u obzir da eksperimentalno detektovani trend promena sekundarnih struktura i pri denaturaciji na niskim temperaturama različito utiče na promenu sadržaja α -heliksa i β -pločica (dosledno: pad α -heliksa i porast β -pločice), navedeno ispitivanje kinetičke stabilnosti papaina moglo bi definisati metodologiju za praćenje stabilnosti papaina na niskoj temperaturi.

Kinetička stabilnost papaina potvrđena je određivanjem konstanti brzina inaktivacije na povišenim temperaturama (tabela 5.2, slika 5.16), kao i računanjem aktivacione energije inaktivacije papaina, koja je iznosila čak 223 ± 6 kJ/mol što je značajno viša vrednost kada se upoređi sa većinom drugih enzima (121 kJ/mol za karbonsku anhidrazu [159], 88 kJ/mol za ureazu [160] i 167 kJ/mol za amilazu [154]). Kada se uzme u obzir da je ficin svrstan u papainsku familiju proteaza na osnovu sličnosti sa papainom [87], kao i na osnovu detektovane nepravilnosti u elektroforetskoj mobilnosti ficina, pretpostavljeno je da bi i ficin mogao predstavljati kinetički stabilan enzim. Iz rezultata ispitivanja kinetičke stabilnosti ficina može se zapaziti da su dobijene vrednosti konstanti brzina inaktivacije ficina uporedive sa prethodno publikovanim vrednostima. Na primer, konstanta brzine termalne inaktivacije ficina na 60 °C određena u ovoj studiji iznosila je $0,015 \pm 0,006$ min⁻¹, dok je u studiji Katsarosa i saradnika određena vrednost od $0,0158 \pm 0,0015$ min⁻¹ na istoj temperaturi [161]. Aktivaciona energija inaktivacije bila je gotovo identična za ficin i papain: 233 ± 4 kJ/mol, odnosno 223 ± 6 kJ/mol. Iz navedenih rezultata, sa sigurnošću se može zaključiti da i ficin spada u kinetički stabilne enzime.

Kriva termalne inaktivacije blago prethodi krivoj denaturaciji papaina (slika 5.15) što ukazuje na to da su i manje strukturne promene dovoljne da inaktiviraju papain. Kriva termalne inaktivacije potvrdila je visoku stabilnost papaina na povišenoj temperaturi. Dobijeni rezultati (detektovana T_{m50} vrednost papaina od 79 ± 2 °C) uporedivi su sa rezultatima dobijenim primenom diferencijalne skenirajuće kalorimetrije (T_m vrednost papaina od oko 83 ± 1 °C) [3, 162]. Sa krive se jasno može uočiti kooperativni gubitak aktivnosti usled termalne denaturacije papaina (slika 5.15). Veća brzina kojom opada procenat aktivne strukture u odnosu na procenat native strukture u opsegu kooperativnog prelaza (slika 5.15) može se objasniti pretpostavljenim nezavisnim rasplitanjem domena papaina [162], te da je gubitak aktivnosti papaina povezan sa kooperativnim prelazom samo jednog od domena. Ostaci aktivnog mesta (Cys-25, His-159 i Asn-178) locirani su u procepu između dva domena, stoga destabilizacija samo jednog od domena može dovesti do inaktiviranja papaina [3].

Pošto ovi rezultati indiciraju da je inaktivacija papaina pod uticajem denaturanata vezana za tranziciju native strukture, tačku promene načina uvijanja labilnog domena papaina (denaturaciju) moguće je pratiti i merenjem aktivnosti.

U cilju opisivanja strukturnih razlika između papaina u prisustvu SDS-a na različitim temperaturama, primenjena je FT-IR spektroskopija, metoda koja se uobičajeno koristi za kvantifikovanje i praćenje promena sekundarnih struktura proteina [142]. Za razliku od ostalih metoda koje se tradicionalno primenjuju za praćenje strukturnih promena proteina, analize strukture FT-IR spektroskopijom moguće je raditi i ukoliko postoje zamućenja u rastvoru, odnosno ukoliko dolazi do agregiranja proteina. Region spektra koji daje najviše informacija kada je sekundarna struktura proteina u pitanju jeste amidni I region (između talasnih brojeva: $1600-1700\text{ cm}^{-1}$) koji predstavlja signal istežuće vibracije C=O grupe u peptidnoj vezi [142]. Nedavne studije pokazale su da se mogu javiti specifične trake niskih frekvencija u drugom izvodu spektra u amidnom I regionu ukoliko proteini pokazuju tendenciju agregiranja [56]. Nekoliko autora je prijavilo da su frekvencije $1614-1624\text{ cm}^{-1}$ i iznad 1685 cm^{-1} frekvencije specifične za formiranje agregata koje se pojavljuju usled formiranja intermolekulskih antiparalelnih β -pločica neposredno pre agregiranja [56, 137, 149]. Kako je, za razliku od amidnog I regiona, amidni II region ($1480-1575\text{ cm}^{-1}$) manje osetljiv na perturbacije strukture, za

procenu sklonosti proteina da pod određenim uslovima zauzme neku sekundarnu strukturu nije neophodno raditi detaljnu analizu spektara, već je moguće upoređivati intenzitete određenih traka iz amidnog I regiona sa intenzitetom amidnog II regiona kao internim standardom [132, 144].

FT-IR spektroskopijom je potvrđeno da do denaturacije papaina u prisustvu SDS-a dolazi tek na temperaturi od 90 °C, dok na 60 °C protein ostaje nativan (slike 5.17-5.19), kao i da ne dolazi do stehiometrijskog vezivanja SDS-a na 60 °C, za razliku od 95 °C o čemu svedoči sporija migracija na SDS-PAGE ukoliko se papain inkubira na 60 °C (prilog 5).

Denaturacija papaina povišenom temperaturom (90°C) dovela je do smanjenja sekundarnih struktura prisutnih u nativnom papainu, i to: α -heliksa (traka na 1654 cm^{-1}) i neuređene strukture (traka na 1643 cm^{-1}) (slike 5.17, 5.18 i 5.19). S druge strane, došlo je do formiranja intermolekulske β -pločice specifične za agregate: oko 1614-1622 cm^{-1} [137, 163] (slika 5.17). Dobijeno povećanje sadržaja β -pločica u saglasnosti je sa literaturom u vezi denaturacije i agregiranja drugih proteina na povišenim temperaturama. Međutim, do formiranja agregata ne dolazi na povišenoj temperaturi (60 i 90 °C, slike 5.18 i 5.19) u prisustvu detergenta (SDS), već dolazi do povećanja sadržaja neuređenih struktura, uz smanjenje α -heliksa. Promene u sadržaju β -pločica u prisustvu SDS-a nisu izražene, što dovodi do zaključka da denaturacija papaina kombinacijom povišene temperature i SDS-a počinje u okviru α -helikoidnog domena, što svedoči o nižoj stabilnosti domena bogatog α -heliksom u odnosu na domen bogat β -pločicama (kao što je pronađeno i u slučaju papaina usled denaturacije na niskim pH vrednostima [164]). Objašnjenje rezistencije kinetički stabilnih proteina bazirano je na dve pretpostavke. Do vezivanja SDS-a dolazi prilikom tranzicije između različitih konformacija proteina, kada polipeptidni niz proteina (delimičnim) razvijanjem i promenom konformacije postaje podložan i dostupan za vezivanje SDS-a, vodeći nakon vezivanja do zaključavanja u konformaciji u kojoj je SDS vezan [143]. S druge strane, kinetički stabilni proteini odlikuju se veoma malom strukturnom fleksibilnošću, koja dovodi do suprimiranja delimičnog razvijanja [165]. Kinetički stabilni proteini, a samim tim i papain, dakle, ne zauzimaju otvorene/fleksibilne konformacije, te su rezistentni na SDS, osim ako se ne obezbedi dovoljno energije u formi toplote. Uz izuzetak papainske

familije proteaza, najveći broj kinetički stabilnih proteina, kao i proteina rezistentnih na SDS, zauzima kompaktni β -pločasti obrazac uvijanja [143]. Kao što se može zaključiti na osnovu dobijenih rezultata, papain nije izuzetak iz ovog pravila. Naprotiv, kinetička stabilnost i rezistencija na SDS pokazane su i za papain, i to upravo u domenu bogatom β -pločicom. Domen bogat α -heliksom podložniji je razvijanju na povišenoj temperaturi u prisustvu SDS-a.

Kako su promene sekundarnih struktura prilikom termalne denaturacije papaina konzistentne sa padom tendencije zauzimanja α -helikoidne strukture u korist agregacionih β -pločica, FT-IR spektroskopija bi mogla da bude korisna metoda za proučavanja strukturnih promena proteina denaturisanih na niskim temperaturama, čak i u prisustvu agregata.

Zbog pomenutih poteškoća u eksperimentalnom proučavanju denaturacije proteina na niskim temperaturama i ograničenja metoda koje se u te svrhe primenjuju, u ovoj studiji pribegnuto je pristupu koji je ekstenzivno primenjivan u farmaceutskim istraživanjima proteinskih terapeutika. Naime, rastvor proteina izlaže se ponovljenim ciklusima zamrzavanja/odmrzavanja nakon kojih se prati uticaj istih na aktivnost i strukturu proteina. Prednost ovakvog pristupa sastoji se u tome što on kombinuje uticaj i niske temperature *per se*, i stresova povezanih sa formiranjem leda na stabilnost proteina [40, 53]. Naime, denaturacija proteina na niskim temperaturama posledica je promena u interakcijama između vode i proteina na niskoj temperaturi [166]. Međutim, do denaturacije tokom zamrzavanja mogu dovesti i drugi faktori kao što je promena koncentracija rastvorenih supstanci usled formiranja leda (lokalno koncentrovanje), kao i promene pH vrednosti u okolini proteina [40].

Stabilnost papaina i ficina na niskim temperaturama do sada nije proučavana. Praćenje aktivnosti papaina i ficina nakon svakog ciklusa zamrzavanja/odmrzavanja pokazalo je da sa svakim ciklusom dolazi do značajnog gubitka aktivnosti (slike 5.20 i 5.26). Kako u naučnoj literaturi postoji opšte verovanje da proteaze u zamrznutoj formi gube aktivnost usled autoproteolize, dok nema ni pomena denaturacije, ispitane su i promene i u primarnoj strukturi, ali i na nivou sekundarne i tercijarne strukture papaina i ficina izazvane zamrzavanjem/odmrzavanjem.

Praćenje autoproteolize papaina i ficina nakon svakog ciklusa zamrzavanja/odmrzavanja vezivanjem boje CBB i reverzno-faznom hromatografijom (slike 5.21 i 5.27) dokazuje da dramatična inaktivacija papaina od 75% nakon šest ciklusa zamrzavanja/odmrzavanja (slika 5.21) i ficina od oko 50% nakon sedam ciklusa zamrzavanja/odmrzavanja (slika 5.27), ne može biti pripisana autoproteolizi. Uzrok tih promena nalazi se, pre svega, u perturbacijama sekundarne strukture, uz veoma mali uticaj autoproteolize.

Primenom FT-IR spektroskopije identifikovane su i kvantifikovane trake amidnog I regiona specifične za agregiranje u spektrima papaina denaturisanog visokom, ali i niskom temperaturom, kao i u spektru ficina denaturisanog niskom temperaturom (slike 5.22, 5.23, 5.28 i 5.29). Slične trake detektovane su, međutim, i u spektru nativnog papaina, ali sa zastupljenošću od svega 6,2% u odnosu na 21,3% i 46,2% celokupne površine ispod amidnog I regiona u slučaju papaina denaturisanog visokom, odnosno niskom temperaturom (tabela 5.4). Kod ficina je takođe kvantifikovano povećanje sadržaja intermolekulskih β -pločica specifičnih za agregiranje sa 10,5% kod nativnog na čak 30,5% nakon izlaganja niskoj temperaturi (tabela 5.7).

Prisustvo agregata papaina i ficina formiranih usled skladištenja na niskoj temperaturi potvrđeno je analitičkom gel-filtracijom (slike 5.24 i 5.30). Svega 10% nativnog papaina, odnosno 17% nativnog ficina može se detektovati u hromatogramima ovih proteaza skladištenih na niskoj temperaturi čime se može objasniti tako dramatični gubitak aktivnosti nakon šest/sedam ciklusa zamrzavanja/odmrzavanja (slike 5.20 i 5.26).

Teorija koja stoji iza denaturacije proteina u zamrznutom stanju objašnjava destabilizaciju nativne strukture proteina kolapsom hidrofobnog efekta i slabljenjem hidrofobnih interakcija usled pada temperature što dovodi do izlaganja nepolarnih ostataka vodi i time finalno dovodi do alternativnog načina uvijanja proteina [167]. Nekoliko autora prijavilo je porast sadržaja β -pločice u proteinima denaturisanim niskom temperaturom u poređenju sa nativnim [149, 168]. Kompjuterske simulacije pokazale su da formiranje β -pločica može biti uzrokovano termodinamički povoljnim uspostavljanjem tankih slojeva vode oko nepolarnih ostataka na niskim temperaturama. Ovi tanki slojevi vode mogu da se akomodiraju između struktura u formi β -pločice, ali

ne i α -heliksa [169, 170]. Denaturisani proteini, stoga, usled niske temperature teže da formiraju β -pločice koje predstavljaju glavne gradivne elemente agregata [55], što je i u ovoj studiji pokazano na cistein-proteazama, papainu i ficinu.

Denaturacija i agregiranje proteina usled skladištenja na niskim temperaturama, naročito onih od komercijalnog interesa, od naročite je važnosti za industriju zasnovanu na polipeptidima [171]. Uprkos mišljenju da je gubitak aktivnosti proteaza prilikom čuvanja na niskim temperaturama prouzrokovao autoproteolizom, u ovom radu nedvosmisleno je pokazano da usled čuvanja na niskoj temperaturi papain i ficin podležu izrazitim strukturnim perturbacijama i agregiranju nastalih nenativno uvijenih proteina. Ovi rearanžmani u strukturi papaina i ficina koji su u saglasnosti sa drugim proteinima denaturisanim niskom temperaturom, odgovorni su za gubitak aktivnosti papaina i ficina, u daleko većoj meri od autoproteolize.

Detaljnijom analizom traka na niskim talasnim brojevima amidnog I regiona FT-IR spektara proteina, utvrđeno je da se talasni brojevi razlikuju u zavisnosti od tipa agregata. Naime, obrazac vodoničnih veza, a time i precizan talasni broj, zavisi od broja lanaca u okviru β -pločice, kao i izvijanja β -pločica [172]. U literaturi se porast agregacije β -pločice na talasnim brojevima $1611\text{--}1633\text{ cm}^{-1}$ najčešće pripisuje amiloidnom tipu agregata [172, 173]. Nastajanje konformacije amiloidnih agregata moguće je samo pod strogo kontrolisanim uslovima (koncentracija proteina, pH vrednost, jonska sila, temperatura), jer različiti uslovi mogu dovesti do formiranja određenog tipa agregata (nativni oligomeri, amorfni agregati, amiloidni fibrili) [34]. U nekoliko nedavnih studija dobijeni su amiloidni agregati različitih proteina [172]. U slučaju rekombinantnog transtiretina β -pločicama koje grade amiloidne aggregate dodeljen je talasni broj 1615 cm^{-1} [172], dok je u slučaju amiloidnih agregata α -sinukleina, odnosno insulina, dodeljen talasni broj 1628 cm^{-1} , odnosno 1633 cm^{-1} [174]. Za amorfne aggregate (inkluziona tela) prijavljeni su talasni brojevi od oko $1623\text{--}1625\text{ cm}^{-1}$ [174]. S druge strane, postoje literaturni navodi da se niski talasni brojevi ($1611\text{--}1620\text{ cm}^{-1}$) ne mogu striktno pripisati agregatima. Naime, talasni broj oko 1611 cm^{-1} pripisuje se i apsorpciji karboksilnog bočnog ostatka [175], dok je apsorpcija u opsegu $1624\text{--}1642\text{ cm}^{-1}$ pripisana i nativnoj β -pločici [147, 176].

Agregiranje koje je u ovom radu detektovano kao posledica izlaganja niskim temperaturama, nije amiloidnog tipa kod ficina, koji predstavlja smešu formi (slika 5.11). S obzirom na činjenicu da je obrazac strukturnih rearanžmana kojima ficin podleže detektovan i kod papaina, najverovatnije da ni agregati papaina nisu amiloidnog tipa.

Kako do destabilizacije papaina dolazi u okviru α -helikoidnog domena, a ficin ima slične strukturne karakteristike kao papain, verovatni mehanizam agregiranja ove dve cistein-proteaze podrazumeva formiranje β -pločica na mestu α -helikoidnih struktura, čime hidrofobni ostaci, zbog rearanžmana bivaju izloženi na površini proteina vodeći hidrofobnom agregiranju.

Kako je za inaktiviranje ispitivanih cistein-proteaza usled izlaganja niskim temperaturama odgovorna denaturacija, a ne autoproteoliza, postavljena je radna hipoteza da bi sličan fenomen mogao da se dešava i kod serin-proteaza.

Tripsin za sekvenciranje predstavlja veoma važan komercijalni enzim koji nalazi široku primenu u istraživanju. Iako je veoma dobro opisan u literaturi i okarakterisan, ovaj komercijalno važan enzim nije dovoljno ispitan u kontekstu stabilnosti na niskoj temperaturi. Već decenijama se pribegava propisanom načinu skladištenja tripsina za sekvenciranje na niskoj temperaturi. Proizvođači tripsina za sekvenciranje preporučuju čuvanje rastvora tripsina u zamrznutom obliku nakon rastvaranja u 1 mM HCl. Pošto na pH vrednosti 3, tripsin zauzima stanje stopljene globule i nije aktivan [94], ovakav način čuvanja ograničen je na mali broj ciklusa zamrzavanja/odmrzavanja. Upravo zbog nepostojanja adekvatnog načina čuvanja tripsina u rastvornom obliku, kao i zbog njegovog komercijalnog značaja, tripsin je odabran kao jedan od model sistema za ispitivanje stabilnosti na niskim temperaturama, ali i za nalaženje optimalnih uslova za njegovo skladištenje u rastvornom obliku.

Postavljena je hipoteza da bi tripsin trebalo da bude konformaciono stabilniji na svom operativnom pH (pH oko 8,0) pri čuvanju na niskoj temperaturi, nego na pH vrednosti koja je izrazito kisela (pH 3,0). Da bi se testirala postavljena hipoteza, upoređen je način skladištenja tripsina koji preporučuju proizvođači (u kiseljoj sredini, pH 3,0) sa skladištenjem tripsina u alkalnoj sredini (100 mM AB, pH 8,0). Praćenjem aktivnosti

tripsina nakon svakog ciklusa zamrzavanja/odmrzavanja dobijen je preko 2,5 puta veći procenat rezidualne aktivnosti na kraju sedmog ciklusa ukoliko je tripsin zamrzavan u amonijum-bikarbonatu (slika 5.31). U slučaju amonijum-bikarbonata je, osim rada na pH vrednosti na kojoj je protein nativan, najverovatnije na aktivnost tripsina pozitivno uticao i stabilizujući kozmotropski efekat bikarbonatnih jona [177].

Iako je skladištenje tripsina u amonijum-bikarbonatu dalo mnogo bolje rezultate od čuvanja u kiselim uslovima, dodatni cilj bio je da se primenom poznatih krioprotektivnih aditiva optimizuju uslovi čuvanja tripsina i time dodatno poveća procenat očuvanja aktivnosti. Sve odabrane klase krioprotektanata dovele su do još bolje prezervacije aktivnosti tripsina na niskim temperaturama, pošto je izmeren procenat rezidualne aktivnosti iznosio 98, odnosno 99% u slučaju glicerola, odnosno lizina na kraju sedmog ciklusa zamrzavanja/odmrzavanja (slike 5.32 i 5.33). Polioli i aminokiseline predstavljaju dobro poznate stabilizatore proteina u rastvoru. Bez obzira što pripadaju hemijski različitim klasama jedinjenja, za sve krioprotektante je mehanizam stabilizovanja strukture proteina zajednički. Naime, otkriveno je da su ova jedinjenja potisnuta sa površine proteina favorizujući pritom uvijeno stanje. Imaju sposobnost da štite površinu proteina od stresova izazvanih zamrzavanjem kao što su promene u koncentraciji rastvorenih soli, promene u pH vrednosti mikrookoline i temperature ispod 0 °C [178]. Čak i PEG koji se vezuje za proteine na višim temperaturama (>25°C), biva potisnut sa površine proteina istim mehanizmom usled slabljenja hidrofobnih interakcija na niskim temperaturama [42], te takođe štiti tripsin tokom zamrzavanja (slika 5.32). Za iste klase jedinjenja pokazano je da povećavaju procenat očuvanja aktivnosti proteina nakon liofilizacije [144, 179].

Za dalji tok eksperimenata odabrani su 100 mM amonijum-bikarbonat koji sadrži 0,3 M glicerol, odnosno 0,1 M lizin kao stabilizatori koji su dali najveći procenat očuvanosti aktivnosti tripsina i koji, dodatno, ne ometaju tehnike proteomike u kojima se komercijalni tripsin primarno upotrebljava.

Nakon optimizovanja sastava pufera, ispitane su strukturne promene tripsina koje se dešavaju usled ponovljenih ciklusa zamrzavanja/odmrzavanja. Promene na primarnoj strukturi (uzrokovane autoproteolizom) nisu detektovane u tripsinu zamrzavanom u kiselim uslovima, usled toga što se smanjivanjem pH vrednosti ispod 4 tripsin inaktivira

kao posledica protonovanja aspartata u aktivnom centru (čija je pKa vrednost oko 3) [180]. Stoga, autoproteoliza ne može objasniti gubitak aktivnosti od oko 70% posle 7 ciklusa zamrzavanja/odmrzavanja (slika 5.31). S druge strane, ukoliko se tripsin izlaže ciklusima zamrzavanja/odmrzavanja na svojoj operativnoj pH vrednosti (u amonijum-bikarbonatu) detektovano je manje od 10% autoproteolize komercijalnog preparata nakon 7 ciklusa zamrzavanja/odmrzavanja (slika 5.34) o čemu svedoči i postojanje očuvanog pika koji potiče od tripsina u gel-filtraciji visokih performansi (slika 5.35). Takođe, u nekim ranijim studijama izmereno je da je autoproteoliza tripsina na pH 8 i 25 °C ograničena na nekoliko procenata u prvih 10 minuta merenja [181, 182]. Stoga se nameće logičan zaključak: bez obzira na smanjenu brzinu autoproteolize tripsina na pH 3,0, čini se da kiseli uslovi čuvanja tripsina ipak nisu optimalni kada se uzme u obzir pad aktivnosti od oko 9% po ciklusu zamrzavanja/odmrzavanja (slika 5.31). Osim toga, kod tripsina zamrzavanog u amonijum-bikarbonatu sa dodatkom glicerola, odnosno lizina, gubitak aktivnosti (1-2%) (slike 5.32 i 5.33), kao i stepen autoproteolize (do 7%) (slika 5.34) posle 7 ciklusa zamrzavanja i odmrzavanja ograničeni su na svega nekoliko procenata. Navedeni rezultati ukazuju da se potencijalni uzrok detektovanog pada aktivnosti tripsina skladištenog u kiselim uslovima, pre može pripisati denaturaciji na niskim temperaturama nego autoproteolizi.

Promene u FT-IR spektru usled zamrzavanja/odmrzavanja tripsina u kiselim uslovima svedoče o povećanju strukture β -pločice, kao i padu sadržaja neuređenih struktura i α -heliksa (slike 5.36 i 5.38, tabela 5.9). Poređenjem spektara tripsina na pH 3,0 i na pH 8,0 može se zaključiti da dolazi do određenih konformacionih promena čak i bez zamrzavanja i odmrzavanja, već samo usled inkubacije tripsina u kiseloj sredini. Ipak, izraženije razlike u strukturi u odnosu na uzorke u alkalnoj sredini nastupaju kada se tripsin zamrzava/odmrzava u kiseloj sredini (slike 5.36-5.39). Slični spektri sa tri najizraženija maksimuma u regionu 1630-1660 cm^{-1} snimljeni su za tripsin u kiselim uslovima [183], dok su u spektru snimljenom na približno neutralnoj pH vrednosti identifikovana dva maksimuma [184]. Rezultati izračunatih sadržaja sekundarnih struktura takođe potvrđuju da do najvećih strukturnih promena dolazi u uzorku tripsina koji je zamrzavan/odmrzavan u kiseloj sredini (tabela 5.9), koji se pritom i najviše razlikuje od sadržaja sekundarne strukture nativnog tripsina izračunatih na osnovu trodimenzionalne strukture tripsina iz baze podataka UniProt.

Do denaturacije ne dolazi kod uzoraka tripsina čuvanih u baznoj sredini sa krioprotektantima, sudeći po sekundarnim strukturama (slike 5.37 i 5.39). Rezultati nisu iznenađujući jer faktori koji stabilizuju proteine u vodenom rastvoru ispoljavaju isti efekat tokom zamrzavanja/odmrzavanja [178]. Stoga, skladištenje tripsina na svojoj operativnoj pH vrednosti, u prisustvu stabilizujućih aditiva, daje najveće očuvanje strukture proteina, kao što je pokazano i u slučaju himotripsinogena [185] i laktat-dehidrogenaze [186].

Korelacioni koeficijenti spektara ukazuju na to da je očuvanost sekundarnih struktura najviša u uzorcima tripsina zamrzanim/odmrzanim u amonijum-bikarbonatu sa glicerolom, odnosno lizinom (tabela 5.10). Sličan pristup iskorišćen je u studiji Prestrelskog i saradnika koji su pokazali da aditivi za krioprotekciju mogu značajno da povećaju koeficijent korelacije liofilizovane i rehidratisane laktat-dehidrogenaze u poređenju sa kontrolnim uzorkom [138].

Za razliku od cistein-proteaza, kod tripsina nije detektovano značajno povećanje procenta agregacione β -pločice (tabela 5.9). Apsorpcija koja potiče od inter- ili intramolekulske β -pločice slična je i u nativnom tripsinu i u tripsinu koji je zamrzavan/odmrzavan u kiseloj sredini, što ukazuje na to da je najverovatnije u pitanju intramolekulska antiparalelna β -pločica koja postoji i u nativnom tripsinu [96] i da ne dolazi do agregiranja. Odsustvo agregiranja tripsina potvrđeno je gel-filtracijom visokih performansi u kojoj nije dobijen pik koji odgovara većim molekulskim masama od tripsina (slika 5.35).

Uzevši u obzir prikazane rezultate, može se sa sigurnošću tvrditi da tripsin podleže strukturnim promenama analognim onima koje su zabeležene kod drugih proteina koji imaju nativni centralni β -način uvijanja posle denaturacije na niskim temperaturama [7, 168]. Znači da je denaturacija tripsina na niskim temperaturama odgovorna za oko 70% izgubljene aktivnosti tripsina rastvorenog u HCl nakon 7 ciklusa zamrzavanja/odmrzavanja (slika 5.31).

O razlici u stabilnosti tripsina izlaganog niskoj temperaturi u kiseloj i blago baznoj sredini svedoče i različite aktivacione energije inaktivacije koje po ciklusu zamrzavanja/odmrzavanja iznose $21,4 \pm 0,2$ kJ/mol u kiseloj sredini, odnosno $24,6 \pm 0,2$

kJ/mol u baznoj (optimalnoj) sredini. Dobijeni rezultati, stoga, indiciraju da je denaturacija detektovana u kiseloj sredini, a ne i u baznoj, posledica kombinovane destabilizacije usled niske pH vrednosti i temperature. Kako je u literaturi pokazano, niska pH vrednost predstavlja okidač za formiranje stanja stopljene globule [187-190], intermedijera od koga dalje može doći do denaturacije na niskim temperaturama, kao što je pokazano u slučaju predominantno β -pločastog proteina, β -laktoglobulina [191].

Da bi se ispitala funkcionalnost različito tretiranih uzoraka tripsina u metodi za koju je primarno namenjen, uzorci su upoređeni i po parametru efikasnosti u digestiji BSA i identifikaciji metodom otiska prsta. Tripsin u kiseloj sredini (nakon 7 ciklusa zamrzavanja/odmrzavanja) nije se pokazao kao funkcionalan (BSA nije detektovan), za razliku od tripsina u kiseloj sredini koji nije zamrzavan i tripsina u alkalnoj sredini sa krioprotektantima (nakon 7 ciklusa zamrzavanja/odmrzavanja) (tabela 5.11), što još jednom pokazuje da je optimalan način čuvanja tripsina u alkalnoj sredini sa krioprotektantima. Osim toga, odsustvo pojedinih autoproteolitičkih peptida tripsina skladištenog u amonijum-bikarbonatu sa lizinom (prilog 8), svedoči da je hipoteza o supresiji aktivnosti tripsina lizinom tačna. Takođe, u studiji Čamrada i saradnika pokazano je da prisustvo arginina dovodi do značajnog pada u autoproteolizi tripsina [192]. Moguće je da i arginin i lizin inhibiraju autoproteolizu tripsina okupirajući mesto vezivanja supstrata, ali bez prouzrokovanja dramatično negativnog efekta na TMF (tabela 5.11) uzimajući u obzir trajanje procesa digestije tokom pripreme uzoraka za TMF [192]. Kao zaključak ispitivanja stabilnosti tripsina na niskoj temperaturi nameće se da na očuvanost aktivnosti tripsina utiču dva faktora: denaturacija proteina i autoproteoliza. Međutim, bez obzira na izraženu tendenciju tripsina da autoproteolizuje, detektovan je veoma mali procenat autoproteolize u amonijum-bikarbonatnom puferu uz izbegavanje duže manipulacije na sobnoj temperaturi između ciklusa zamrzavanja/odmrzavanja. Denaturacija proteina, kao faktor za koji je u ovoj studiji pokazano da najviše doprinosi gubitku aktivnosti tripsina, može se inhibirati u blago alkalnim uslovima uz dodatak krioprotektanata, i time postići duži vek trajanja tripsina za sekvenciranje.

Biohemijska karakterizacija novootkrivene kolagenaze lateksa smokve pokazala je da je reč o veoma stabilnom enzimu na povišenoj temperaturi i u širokom intervalu pH

vrednosti (prilog 1). Stabilnost na niskoj temperaturi takođe je ispitana u ovoj studiji, s obzirom na veoma visoki potencijal primene ovog enzima u biotehnologiji i medicini. Naime, povoljne karakteristike novootkrivene kolagenaze, osim dostupnosti iz lateksa i jednostavnog postupka izolovanja, jesu relativno visoka aktivnost (u poređenju sa komercijalnom kolagenazom iz bakterijskog soja *Clostridium histolyticum*), visoka supstratna specifičnost (za želatin i kolagen) (slike 5.1-5.3 i 5.9 i 5.10), niska opšta proteolitička aktivnost (slika 5.3), kao i već pomenuta temperaturna i pH stabilnost (prilog 1).

Pristupom uzastopnog zamrzavanja/odmrzavanja ispitane su promene u aktivnosti kolagenaze nakon svakog ciklusa. Denzitometrijska analiza zimograma kolagenaze pokazala je da aktivnost ostaje očuvana nakon svih sedam ciklusa (slika 5.40), što ukazuje na to da kolagenaza nije inaktivirana niskom temperaturom. Ispitano je postojanje potencijalnih promena na primarnoj strukturi kolagenaze nakon svakog ciklusa usled autoproteolize, međutim, primarna struktura ostaje očuvana (slika 5.41). Perturbacije sekundarnih struktura kolagenaze ispitane su FT-IR spektroskopijom, ali nisu detektovane statistički značajne promene (slike 5.42 i 5.43, tabela 5.13). Za kolagenazu ne postoje strukturni podaci u literaturi, međutim, i kod tripsina su strukturne perturbacije detektovane samo uz destabilizaciju strukture kiselim uslovima koji najverovatnije već unapred iniciraju formiranje stanja stopljene globule. Iz dobijenih rezultata proizilazi da su i tripsin i kolagenaza stabilni enzimi na niskoj temperaturi, na operativnoj pH vrednosti.

Kada se uzmu u obzir svi ispitani model sistemi, ističe se suštinska razlika u mehanizmu denaturacije između ispitivanih serin-proteaza (tripsina i kolagenaze) sa jedne, i cistein-proteaza (papaina i ficina) sa druge strane.

Kod tripsina, proteina sa dominantnim sadržajem β -pločica, detektovano povećanje sadržaja intramolekulske β -pločice ukazuje na to da dolazi do takvog strukturnog rearanžmana koji podrazumeva prelaz u regionu jednog ili oba spoljno orijentisana heliksa i neuređenih struktura u β -pločice, ali uz izostanak formiranja nukleusa agregacije, pošto nije detektovana intermolekulska β -pločica (slika 5.44). Predloženi mehanizam denaturacije tripsina na niskim temperaturama u skladu je sa rezultatima koje su dobili Matsuo i saradnici u slučaju tioredoksina [168] i Luan i saradnici [7] u

slučaju C-terminalnog domena ribozomalnog proteina L9 (CTL9). Kao i tripsin koji je ispitivan u ovom radu, i tioredoksin i CTL9 imaju slučan obrazac uvijanja (jezgro izgrađeno od β -pločica i α -helikse spolja).

Papain, s druge strane, najverovatnije podleže suštinski drugačijem strukturnom rearanžmanu koji dovodi do kolapsa manje stabilnog α -helikoidnog domena (koji u slučaju papaina predstavlja mnogo veći udeo u ukupnim sekundarnim strukturama, nego kod tripsina), time formirajući nukleus za nenativno uvijanje sa predominantnim sadržajem intermolekulske β -pločice (slika 5.44). Takođe, detektovane promene u protoku toplote u regionu krive denaturacije ispod 40 °C (slika 5.14B) ukazuju da dolazi do povećane izloženosti nepolarnih bočnih ostataka vodi prilikom izlaganja rastvora papaina niskoj temperaturi, prateći isti obrazac kao prilikom denaturacije β -laktoglobulina na niskim temperaturama [191]. Kao zaključak nameće se da tokom denaturacije papaina i ficina i prelaska α -helikoidnog domena u β -pločice dolazi do izlaganja mnogo većeg udela hidrofobnih ostataka koje su se nalazili u unutrašnjosti proteina na površinu novoformiranih β -pločica, koje posledično služe kao nukleusi agregacije. Slično ponašanje prilikom denaturacije na niskim temperaturama uočeno je i kod α -helikoidnog proteina mioglobina [149].

7. ZAKLJUČCI

Gubitak aktivnosti proteaza tokom skladištenja na niskoj temperaturi, ne može se pripisati autoproteolizi, već predstavlja, u najvećoj meri, posledicu denaturacije proteina, koja može biti praćena i agregiranjem nenativnih proteina.

a. Izolovanje enzima koji su predstavljali model sisteme ove studije

1) Kolagenaza i ficin iz lateksa smokve (*F. carica*) mogu biti paralelno prečišćeni procedurom koja podrazumeva kombinaciju gel-filtracije, kovalentne hromatografije i jonoizmenjivačke hromatografije.

2) Kolagenaza lateksa smokve nije ranije detektovana, niti opisana u naučnoj literaturi. Na osnovu bazične karakterizacije kolagenaze zaključeno je da je reč o monomernom proteinu molekulske mase oko 41 kDa i pI vrednosti oko 5. Kolagenaza pripada klasi serin-proteaza i specifična je za kolagen i želatin. Karakteriše je niska opšta proteolitička aktivnost. Reč je o veoma stabilnom enzimu široke pH i temperaturne stabilnosti. Optimalno je aktivna na pH vrednosti oko 8-9 i na temperaturi oko 60 °C.

Uzevši u obzir sve navedene karakteristike kolagenaze, zaključeno je da ovaj enzim ima potencijal za primenu u različitim oblastima biotehnologije, medicine i nauke.

b. Optimizovanje metodologije za ispitivanje stabilnosti serin- i cistein-proteaza na niskim temperaturama

1) Ispitivanjem stabilnosti papaina u širokom temperaturnom intervalu, korišćenjem konvencionalnih metoda za ispitivanje denaturacije proteina, zaključeno je da se promene strukture ne mogu pratiti na dovoljno niskim temperaturama (ispod tačke mržnjenja vode), već da se rezultati u regionu niskih temperatura mogu dobiti jedino ekstrapoliranjem rezultata iz oblasti viših temperatura (iznad 0 °C).

2) Na osnovu ispitane kinetičke stabilnosti i inaktivacije papaina, zaključeno je da je inaktiviranje papaina pod uticajem denaturanata vezano za tranziciju native strukture, te da je tačku promene načina uvijanja papaina (denaturaciju) moguće pratiti i merenjem aktivnosti.

3) Zaključeno je i da je praćenje promena sekundarnih struktura usled izlaganja niskoj temperaturi moguće primenom infracrvene spektroskopije, čak i u slučaju formiranja agregata.

c. Ispitivanje stabilnosti serin- i cistein-proteaza na niskim temperaturama

1) Cistein-protease (papain i ficin) podležu intenzivnom padu aktivnosti usled izlaganja niskim temperaturama, dok u slučaju serin-proteaza (tripsin i kolagenaza) aktivnost ostaje očuvana na operativnoj pH vrednosti ovih enzima. Do pada aktivnosti tripsina dolazi tek usled kombinovanog efekta niske temperature i niske pH vrednosti.

2) Ispitivanjem stabilnosti primarne strukture proteaza, zaključeno je da se detektovano smanjenje aktivnosti proteaza ne može pripisati autoproteolizi, jer primarna struktura svih ispitivanih proteaza ostaje gotovo potpuno očuvana nakon izlaganja niskim temperaturama.

3) Na osnovu kvantifikovanih promena na nivou sekundarnih struktura, može se zaključiti da usled izlaganja niskim temperaturama dolazi do strukturnih rearanžmana karakterističnih za denaturaciju u slučaju papaina, ficina i tripsina (na pH 3). Tripsin (pH 8) i kolagenaza su konformaciono stabilni na niskim temperaturama, pošto ne podležu značajnim strukturnim promenama.

4) U slučaju cistein-proteaza, papaina i ficina, denaturacija izazvana niskim temperaturama praćena je formiranjem agregata.

d. Optimizovanje uslova za skladištenje tripsina za sekvenciranje u rastvornom obliku na niskoj temperaturi

1) Ispitivanjem promena aktivnosti i strukture tripsina nakon izlaganja niskim temperaturama, zaključeno je da ukoliko se tripsin izlaže niskim temperaturama u blago alkalnoj sredini uz dodatak glicerola ili lizina dolazi do prezerviranja aktivnosti i suprimiranja denaturacije na niskim temperaturama, čime se produžava vek trajanja tripsina.

2) Tripsin izlagan niskim temperaturama u blago alkalnoj sredini uz prisustvo glicerola ili lizina jednako je funkcionalan u metodi peptidnog mapiranja kao i netretirani tripsin.

8. LITERATURA

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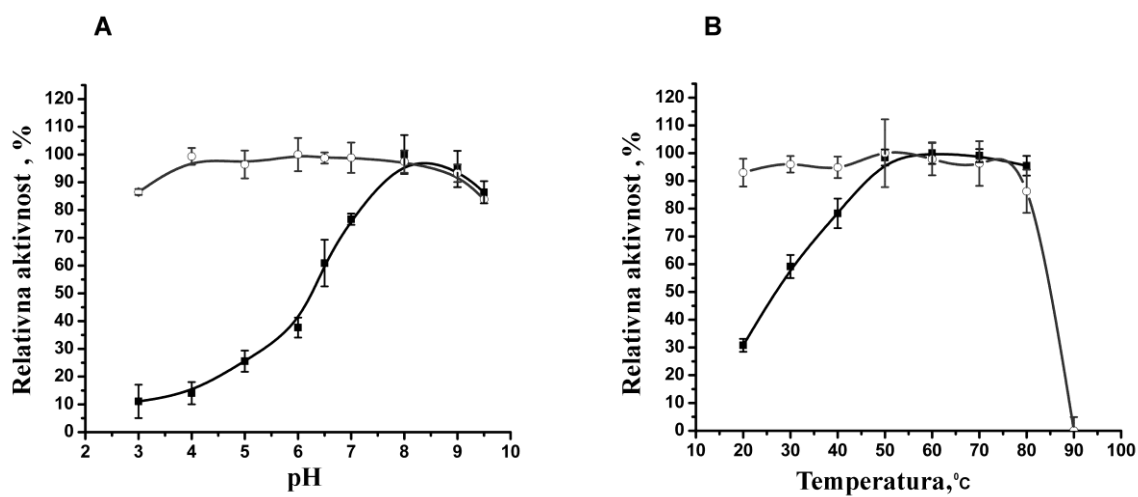
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PRILOZI

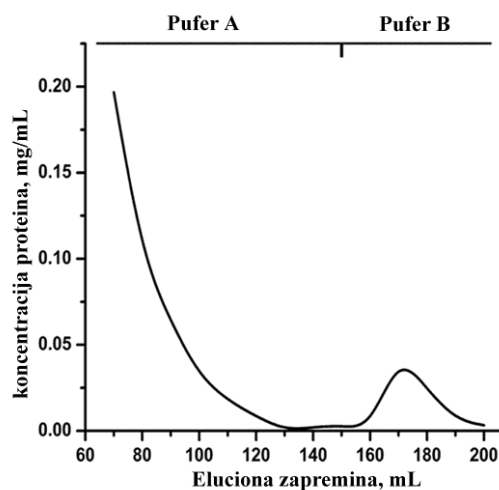
PRILOG 1. A) pH stabilnost i optimum; B) temperaturna stabilnost i optimum kolagenaze lateksa smokve. Prazni kvadratići označavaju stabilnost, dok popunjeni označavaju optimume.



PRILOG 2. Tabela prečišćavanja papaina.

	Aktivnost (U/mL)	Koncentracija proteina, (mg/mL)	Specifična aktivnost, (U/mg)	Stepen prečišćenosti
Sirovi ekstrakt	83,3 ± 0,5	15,3 ± 0,2	1,13	1
Taloženje acetonom	73 ± 1	13,6 ± 0,5	1,12	0,99
Kovalentna hromatografija	3,9 ± 0,1	0,091 ± 0,002	9,0	7,9

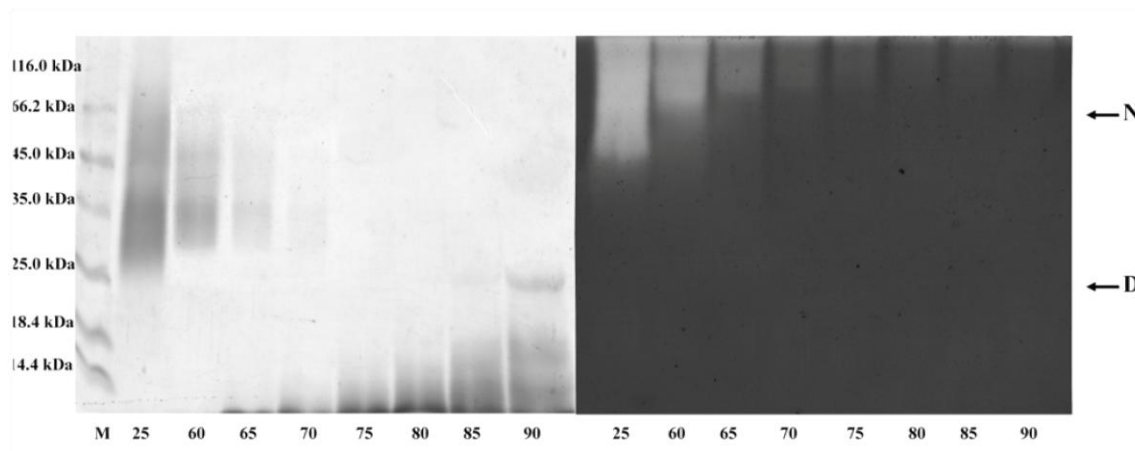
PRILOG 3. Hromatografsko prečišćavanje papaina na tiol-Sepharose matriksu za kovalentnu hromatografiju. Kolona je ekvilibrisana i nevezani proteini eluirani 0,1 M Tris puferom pH 8,0 (pufer A), a potom su vezani proteini eluirani istim puferom sa 20 mM L-cisteinom (pufer B). Frakcije su sakupljene pod protokom od 0,8 mL/min.



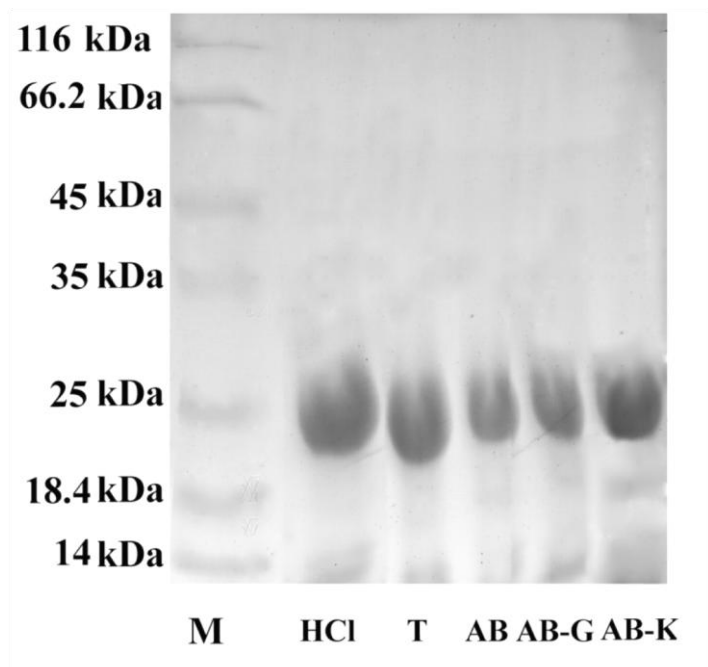
PRILOG 4. TMF analiza izolovanog papaina sa naglašenim identifikovanim peptidima.

1 IPEYVDWR**QK GAVTPVKNQG** SCGSCWAFSA VVTIEGIIKI RTGNLNEYSE
51 QELLDCDRRS YGCNGGYPWS ALQLVAQYGI HYR**NTYPYEG VQRYCRSREK**
101 **GPYAAKTDGV RQVQPYNEGA** LLYSIANQPV SVVLEAAGK**D FQLYRGGIFV**
151 GPCGNKVDHA VAAVGYGPNY ILIKNSWGTG WGENGYIRIK**R**GTGNSYGVC
201 GLYTSSFYPV KN

PRILOG 5. SDS-PAGE i zimogramska analiza (prema želatinu kao supstratu) papaina inkubiranog na 25 °C, kao i na povišenim temperaturama (60-90 °C) u prisustvu 1% SDS-a. N - nativni papain; D - denaturisani papain.



PRILOG 6. SDS-PAGE različito tretiranih uzoraka tripsina: HCl - netretirani - tripsin rastvoren u 1 mM HCl; uzorci nakon sedam ciklusa zamrzavanja/odmrzavanja: T - tripsin rastvoren u 1 mM HCl; AB - tripsin rastvoren u 100 mM amonijum-bikarbonatu; AB-G - tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,3 M glicerolom; AB-K - tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,1 M lizinom.



PRILOG 7. Lista identifikovanih peptida specifičnih za BSA u uzorcima digestovanim sa različito tretiranim tripsinom (netretirani - tripsin rastvoren u 1 mM HCl; HCl - tripsin rastvoren u 1 mM HCl nakon sedam ciklusa zamrzavanja/odmrzavanja; AB-G - tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,3 M glicerolom; AB-K - tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,1 M lizinom).

Uzorak tripsina	Početak-kraj	Peptid
HCl; AB-K	29-36	SEIAHRFK
AB-K	66-88	LVNELTEFAKTCVADESHAGCEK
Netretirani; HCl	76-88	TCVADESHAGCEK
AB-K	89-100	SLHTLFGDELCK
Netretirani; AB-G; AB-K	101-105	VASLR
Netretirani; HCl; AB-K	106-117	ETYGDMADCCEK
Netretirani	118-122	QEPER
Netretirani; HCl; AB-G; AB-K	131-138	DDSPDLPK
Netretirani	139-156	LKPDPNTLCDEFKADEKK
AB-G; AB-K	152-160	ADEKKFWGK
Netretirani; AB-G; AB-K	157-160	FWGK
Netretirani	161-167	LYEIIAR
Netretirani; AB-G; AB-K	198-204	GACLLPK
AB-G	219-222	QRLR
Netretirani; AB-G; AB-K	223-228	CASIQK
Netretirani; AB-G; AB-K	229-232	FGER
Netretirani; AB-G	236-241	AWSVAR
AB-G	242-245	LSQK
Netretirani; AB-G; AB-K	246-248	FPK
AB-G; AB-K	246-266	FPKAEFVEVTKLVTDLTQVHK
Netretirani; HCl; AB-G; AB-K	249-256	AEFVEVTK
Netretirani; AB-G; AB-K	257-263	LVTDLTK
Netretirani; AB-K	264-266	VHK
Netretirani	264-280	VHKECCHGDLLECADDR
AB-G	281-285	ADLAK
AB-G	281-297	ADLAKYICDNQDTISSK
HCl; AB-K	286-297	YICDNQDTISSK
HCl; AB-G; AB-K	286-318	YICDNQDTISSKLKECCDKPLLEKSHCI AEVEK
HCl	310-318	SHCIAEVEK
HCl; AB-G; AB-K	310-340	SHCIAEVEKDAIPENLPPLTADFAEDKD VCK
Netretirani; AB-G; AB-K	341-346	NYQEAK
Netretirani	361-374	HPEYAVSVLLRLAK
AB-G	372-386	LAKEYEATTLIECCAK
HCl; AB-G	375-386	EYEATTLIECCAK
Netretirani; AB-K	413-436	QNCDQFEKLGEYGFQNALIVRYTR
AB-G	434-436	YTR
Netretirani; HCl; AB-G; AB-K	434-451	YTRKVPQVSTPTLVEVSR
Netretirani; AB-G; AB-K	452-455	SLGK
Netretirani; AB-G	456-459	VGTR
HCl; AB-G; AB-K	469-482	MPCTEDYLSLILNR
HCl	483-489	LCVLHEK

Netretirani; AB-G; AB-K	490–495	TPVSEK
Netretirani	496–498	VTK
Netretirani; AB-G; AB-K	524–528	AFDEK
Netretirani	524–547	AFDEKLFTFHADICTLPDTEKQIK
Netretirani; HCl; AB-G; AB-K	529–548	LFTFHADICTLPDTEKQIKK
Netretirani; AB-K	545–548	QIKK
Netretirani	545–557	QIKKQTALVELLK
HCl	548–557	KQTALVELLK
AB-K	558–561	HKPK
Netretirani; AB-G; AB-K	562–568	ATEEQLK
AB-G; AB-K	569–580	TVMENFVAFVDK
Netretirani; AB-G; AB-K	581–597	CCAADDKEACFAVEGPK
Netretirani; AB-G	588–597	EACFAVEGPK
Netretirani; HCl; AB-G; AB-K	598–607	LVVSTQTAL

PRILOG 8. Peptidi koji potiču od autoproteolize tripsina. Uzorci tripsina korišćeni za digestiju BSA: netretirani - tripsin rastvoren u 1 mM HCl; HCl - tripsin rastvoren u 1 mM HCl nakon sedam ciklusa zamrzavanja/odmrzavanja; AB-G - tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,3 M glicerolom; AB-K - tripsin rastvoren u 100 mM amonijum-bikarbonatu sa 0,1 M lizinom. Peptidi obeleženi zvezdicom nedostaju u AB-K uzorku.

Početak-kraj	Monoizotopska masa (m/z)	Peptid
113-114	260.197	LK
160-162	363.206	CLK
241-246*	633.320	QTIASN
67-72	659.383	SGIQVR
115-122	805.416	SAASLNSR
224-231*	906.504	NKPGVYTK
163-172	1020.503	APILSDSSCK
232-240	1111.560	VCNYVSWIK
149-159	1153.574	SSGTSYPDVLK
194-209	1495.615	DSCQGDSGGPVVCSGK
173-193	2193.994	SAYPGQITSNMFCAGYLEGGK
123-148*	2552.248	VASISLPTSCASAGTQCLISGWGNTK

BIOGRAFIJA

Brankica G. Rašković rođena je 30. marta 1988. godine u Kraljevu. Osnovne studije biohemije završila je na Hemijskom fakultetu Univerziteta u Beogradu 2011. godine sa prosečnom ocenom 9,74. Master studije je završila 2012. godine sa prosečnom ocenom 10,00. Tokom završnih godina osnovnih i master studija bila je nosilac plakete Dositeja i dobitnik istoimene stipendije Ministarstva omladine i sporta Republike Srbije. Zbog postignutih rezultata dodeljena joj je nagrada Srpskog hemijskog društva za izuzetan uspeh tokom studiranja. Doktorske studije na Katedri za biohemiju Hemijskog fakulteta upisala je 2012. godine pod mentorstvom doc. dr Natalije Polović. Tokom doktorskih studija uključena je na projekat 172049 kao stipendista Ministarstva prosvete, nauke i tehnološkog razvoja Republike Srbije (2012-2016 godine).

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Prvi je autor šest i koautor dva rada koji su objavljeni u međunarodnim časopisima. Autor je i osam saopštenja na međunarodnim i domaćim konferencijama.

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Characterisation of general proteolytic, milk clotting and antifungal activity of *Ficus carica* latex during fruit ripening

Brankica Raskovic,* Jelena Lazic and Natalija Polovic



Abstract

BACKGROUND: The physiological role of fig latex is to protect the plant from pathogens. Latex is a rich source of proteases, predominantly ficin. Fig latex also contains collagenolytic protease and chitinolytic enzymes. Our aim was to investigate changes in protein composition, enzyme and antifungal activities of fig latex during fruit ripening.

RESULTS: Comparison of latex samples in different time periods showed a uniform increase of protein concentration in chronological order. The content of collagenolytic protease did not differ significantly in the latex samples, while the content of ficin decreased. Ficin-specific activity towards casein was the highest at the beginning of fruit development (about 80 U mg⁻¹). Specific milk clotting activity increased as well as the abundance of casein band in the clots. Specific chitinolytic activity at the beginning of flowering was 6.5 times higher than the activity in the period when fruits are ripe. Antifungal activity is the most extensive in spring.

CONCLUSION: Ficin forms with different casein specificities are present in different proportions during fruit ripening, which is of importance for applications in the dairy industry. The protection mechanism against insects and fungi, which relies on chitinolytic activity, is the most important in the early phases of flowering and is replaced with other strategies over time.

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Supporting information may be found in the online version of this article.

Keywords: *Ficus carica* latex; ficin; ripening; chitinolytic activity; antifungal activity; milk clotting activity

INTRODUCTION

Ficus carica var. *Brown Turkey* (the common fig) is among the earliest cultivated plant species because of its use as a food and medicine. The curative effects of figs were noticed centuries ago, and today fig fruit is used worldwide not just as food, but also in various forms which are part of traditional medicine.¹

The genus *Ficus* is one of 40 members of the Moraceae family which consists of a large number of different species.² Common characteristic of all *Ficus* species is the presence of latex fluid inside laticiferous cells. Latex is a complex, sticky fluid, milky in appearance. Latex is produced by laticiferous cells and is excreted in the place of injury immediately after wound formation.³

One of the most important physiological roles of latex is protection from pathogen invasion throughout the injured tissue.⁴ Younger parts of the fig tree contain the largest volumes of latex (young shoots, pedicles and leaves), since latex represents their only mechanism of protection. Older parts of the plant have many additional protection strategies, e.g. high polyphenol concentration and toughness, and therefore have a lower latex content.³

The defensive role of latex is achieved by mechanical and biochemical protection. Mechanical protection relies on the ability of latex to coagulate at the site of injury, which is attributed to the presence of *cis*-1,4-polyisoprene, commonly known as rubber. Biochemical protection relies on the physiological action of active compounds.⁴

Latex is a complex mixture of many different types of secondary metabolites and proteins which differ between species and enable them to have specific protection strategies. Some of them are alkaloids, terpenoids, tannins, sterols,⁵ polyphenols,⁶ as well as proteins, e.g. proteases, oxidases, lectins, chitinases, glucosidases and phosphatases: all of them are often found in high concentrations.³ One of the main constituents of latex is rubber and the fig tree is considered as an alternative rubber crop because it generates a large volume of latex, has a fast growth habit and long life.⁷

Proteolytic fractions of latices of the genus *Ficus* predominantly contain cysteine proteases, but there are also other classes of proteases, e.g. aspartic⁸ and serine proteases.⁹

Ficin (EC 3.4.22.3) is a common name for the endoproteolytic fraction of fig latex. It is known that there are multiple forms of this enzyme in fig latex, and all of them belong to the cysteine protease class.¹⁰ Ficin from fig latex is known as an enzyme with milk clotting activity (ancient writings such as the *Iliad* indicate that fig latex can be used for cheese-making), but it also has high proteolytic

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activity over a broad temperature and pH range.^{9,11} Apart from ficin, fig latex also contains the newly described collagenolytic serine protease,⁹ as well as a few recently described chitinolytic enzymes.^{12–14}

Fig latex has been extensively investigated due to its high proteolytic activity. However, time-dependent changes in protein composition of fig latex have never been studied. We hypothesised that variations in protein composition, as well as in caseinolytic, milk clotting and antifungal activity of fig latex during fruit ripening could exist. Testing such a hypothesis would be of great importance for using latex to produce proteases for the dairy industry or to prepare novel antifungal agents.

EXPERIMENTAL

Reagents

Bovine serum albumin (BSA), casein, Coomassie brilliant blue R-250 (CBB R-250), L-cysteine, chitin, *N*-acetyl-D-glucosamine (GlcNAc), were purchased from Sigma–Aldrich (Steinheim, Germany). Unstained protein molecular weight markers were bought from Thermo Scientific (Rockford, IL, USA). All other chemicals were commercial products of analytical grade and were used without further purification.

Collection and preparation of latex samples

Latex was collected from a private orchard in Bar, Montenegro, by simple incision of green fruits of the same fig trees. It was sampled approximately every 2 weeks (from 1 May to 15 August). Each time, 10 mL of the latex fluid was collected from the same fig trees (three in total, each time approximately 10 fruits were used). The latex fluid was poured into 1.5-mL micro-centrifuge tubes and was immediately stored at -20°C until used. The frozen latex was thawed at 4°C and was centrifuged at $10\,000\times g$ at 4°C for 60 min to remove insoluble gum (approximately 200 g kg^{-1} in weight) and other debris.⁸ The supernatants obtained were frozen in liquid nitrogen and stored at -20°C if not assayed immediately.

Determination of protein concentration and electrophoretic profiling of latex proteins

The total protein concentration in latex samples was determined by using the Bradford assay, using BSA as standard.¹⁵ Before loading onto the gel, latex samples were diluted to 0.5 mg mL^{-1} and mixed with five times concentrated reducing sample buffer and heated at 95°C for 5 min. The composition of five times concentrated reducing sample buffer was 60 mmol L^{-1} Tris buffer pH 6.8 containing 250 g kg^{-1} glycerol, 20 g kg^{-1} SDS, 14.4 mmol L^{-1} 2-mercaptoethanol, and 1 g kg^{-1} bromophenol blue. Twenty microlitres of each sample was loaded onto the gel. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 120 g kg^{-1} resolving gel and 40 g kg^{-1} stacking gel was performed in a protein electrophoresis unit GV202 (Bio-Step, Jahnsdorf, Germany) according to the method given by Laemmli.¹⁶

Two gels were run in parallel. The gels were stained with CBB-R250.

Densitometric analysis of each gel was done using Gel-Pro Analyzer 3.1 software (Media Cybernetics, Silver Spring, MD, USA). The abundance of both a collagenolytic serine protease band and ficin band was compared between the samples (L1–L8) and also their relative abundances were calculated within the same lane and presented as a percent of all the bands within the lane.

General proteolytic activity

General proteolytic activity was determined using casein as a substrate at pH 7.0 which is the optimum pH for ficin.¹⁷ All the latex samples prepared as described, were diluted at a 1:200 ratio in 100 mmol L^{-1} sodium phosphate buffer pH 7.0 containing 9 g L^{-1} NaCl [phosphate buffered saline (PBS)]. Fifty microlitres of samples were mixed with $250\text{ }\mu\text{L}$ substrate solution (6.5 g L^{-1} casein in PBS containing 10 mmol L^{-1} L-cysteine). Reaction mixtures were incubated for 30 min at 37°C . The reaction was stopped by adding $60\text{ }\mu\text{L}$ of 50 g L^{-1} trichloroacetic acid (TCA), followed by centrifugation for 15 min at $12\,000\times g$. The absorbance at 280 nm of the supernatants was recorded. Appropriate blanks were prepared for every latex sample. Each enzyme blank was prepared by incubating $50\text{ }\mu\text{L}$ of latex sample for 30 min at 37°C without casein as a substrate. After incubation, samples were mixed with $60\text{ }\mu\text{L}$ of TCA, and then $250\text{ }\mu\text{L}$ buffer was added. A substrate blank was prepared by incubating casein solution with buffer only (no enzyme added) and adding TCA solution after incubation of substrate alone for 30 min at 37°C . The absorbance of both blanks was subtracted from the absorbance of the samples. All measurements were performed in triplicate. One unit of caseinolytic activity was defined as amount of enzyme which leads to an absorbance of 1.0 at 280 nm for 1 min.⁹

Specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U mg^{-1} .

Milk clotting assay

The Berridge coagulation assay was used to determine clotting time. The clotting time was defined as the time interval from the addition of the latex to the milk to the formation of the first white flakes of coagulated casein. The assay was done essentially as described.¹⁸ Briefly, 0.5 mL of 100 g L^{-1} of commercial skimmed milk (containing 5 g kg^{-1} fat before 10 times dilution); containing 10 mmol L^{-1} CaCl_2 was preheated at 37°C . The pH value was previously adjusted at 6.5. Then, $200\text{ }\mu\text{L}$ of the preheated 1:10 diluted latex solution (containing 10 mmol L^{-1} L-cysteine) was added. The reaction was incubated at 37°C . The time was measured until the first flakes appeared.¹⁸ The experiment was done in triplicate. A positive control (milk coagulated using chymosin at a concentration 0.044 mg mL^{-1}) was also included. One unit of milk clotting activity was defined as amount of enzyme needed to coagulate 1 mL of 100 g L^{-1} milk in 1 min at 37°C . Specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U mg^{-1} .

The ratio of milk clotting to general proteolytic activity against casein as a substrate (*C/P* value) was also calculated.

Electrophoretic analysis of milk clotting activity

The milk clot obtained using chymosin or using fig latices as coagulating agents was incubated for an additional hour at 37°C to achieve total precipitation of milk coagulum. After centrifugation at $12\,000\times g$ for 20 min, whey was separated from the precipitate and prepared for SDS-PAGE analysis. SDS-PAGE was performed as previously described.

Chitinase activity

Chitinase activity was evaluated by Schales' procedure as described by Ferrari *et al.*¹⁹ Latex samples were diluted at a ratio 1:2 in PBS. The reaction mixtures were prepared by mixing

300- μ L samples with the same volume of substrate suspension (20 g kg⁻¹ colloidal chitin in 50 mmol L⁻¹ phosphate buffer pH 6.2). The reaction mixture was incubated for 20 h at 37 °C. After incubation, samples were centrifuged at 12 000 $\times g$ for 20 min at 4 °C. One volume of supernatant (250 μ L) was added to the same volume of Schales' reagent (0.5 mol L⁻¹ sodium carbonate and 0.5 g L⁻¹ potassium ferricyanide in water). Reaction mixtures were incubated at 100 °C for 15 min and then centrifuged at 12 000 $\times g$ for 20 min. Supernatants were transferred to a 96-well microtitre plate and the absorbance at 405 nm was measured using a LKB microplate reader 5060-006 (GDV, Rome, Italy). Blanks were prepared by mixing 300 μ L of water with the same volume of substrate suspension and further prepared the same way as for latex samples. All measurements were performed in triplicate.

Different concentrations of *N*-acetyl-D-glucosamine (GlcNAc) (1 mmol L⁻¹ to 50 mmol L⁻¹) were used for construction of the standard curve. One unit of chitinase activity was defined as the amount of enzyme that produces 1 μ mol GlcNAc for 1 min under the conditions described.⁷ Specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U mg⁻¹.

Antifungal activity

For the purpose of measuring antifungal activity, a strain of *Saccharomyces cerevisiae* was used. Inoculum was prepared from glycerol stock using a sterile tip and transferring *S. cerevisiae* cells to yeast tryptone dextrose (YTD) liquid medium and cultures were grown at 28 °C overnight. Inocula were diluted in YTD liquid medium to approximately 10⁵ colony forming units (CFU) and then grown in the presence of latex samples. The incubation was carried out in sterile 96-well microplates and yeast growth was monitored by measuring the absorbance (optical density, OD) at 620 nm. Results were expressed as the percentage of yeast growth compared to the control sample incubated in the appropriate buffer in the absence of latex (100 mmol L⁻¹ sodium phosphate buffer, pH 7.4, added to yeast culture). The mean growth values of triplicates were obtained and then converted to the percentage inhibition of cell growth, as described by Prokopovic *et al.*²⁰

RESULTS AND DISCUSSION

Collection of latex and determination of protein concentration

The period of latex collection and exact time intervals are presented in Table 1. This period includes the complete phase of fruit ripening, from the beginning of flowering until the time the fruit completely ripens. A large number of studies on *F. carica* latex and other plant latices, in which method of collection has been described, emphasised that latex has been exploited from the green parts of plant, while the fruit had still been immature.^{10,21} This prompted us to explore whether there are some variations in the composition of fig latex over time.

To explore the potential differences between protein profiles of latex samples in different time intervals, we first determined total protein concentrations (Table 1).

Quite a uniform increase of protein concentration in chronological order can be seen (Table 1). Gooding suggested that the changes of latex protein content are the consequence of more pronounced dry conditions moving from spring to summer, leading to increased (macro)molecule concentration in latex.²²

Table 1. Total protein concentration of *Ficus carica* latex samples collected on different dates

Latex sample	Date of collection	Protein concentration \pm SD (mg mL ⁻¹)
L1	01 May 2013	16 \pm 3
L2	15 May 2013	18 \pm 1
L3	02 June 2013	21 \pm 8
L4	16 June 2013	26 \pm 9
L5	01 July 2013	28 \pm 3
L6	15 July 2013	33 \pm 1
L7	01 August 2013	37 \pm 9
L8	16 August 2013	41 \pm 2

The values represent means \pm standard deviation of data from three independent experiments.

Despite fig latex proteins being investigated in many studies, starting from commercial crude ficin preparation rather than collected latex, there is not much information about *F. carica* latex available for comparison. However, in the study on *F. carica* latex by Chang *et al.*²³ total protein concentration of the latex collected from the unripe fruit was estimated to be approximately 15 mg mL⁻¹ which is in good agreement with our result for latex collected on 1 May (16 mg mL⁻¹). In the study by Whitaker from 1958, protein concentration estimated by the Lowry method was similar to our result from the second half of July, and it was determined to be 33.6 mg mL⁻¹, although Whitaker and colleagues did not point out the exact period of latex collection.²⁴

Latices of other laticiferous plants are considered to be rich sources of proteins.^{5,25,26} Bearing in mind the protein concentrations in fig latices determined during ripening, fig latex collected in the summer is one of the plant latices with the highest protein content.

Electrophoretic profiles of latex samples

Then, we wanted to characterise and compare our latex samples with regard to protein pattern. To study protein composition in fig latex samples, SDS-PAGE analysis was performed. The results are shown in Fig. 1.

Fig latex protein profiles that we detected in this study contain the same protein bands as described previously.⁷ Two main protein bands could be detected in each sample. A protein band around 25 kDa represents cysteine protease ficin, while a band around 48 kDa can be assigned to recently described collagenolytic serine protease.⁹ Additionally, there are several components of molecular weight of 20 kDa and 17 kDa and less than 14 kDa.

Taking into consideration that the protein profile of latex samples did not change significantly during time regarding the presence of main protein bands (Fig. 1), we sought to examine the contents of bands attributed to collagenolytic serine protease and ficin by more detailed densitometric analysis. The analysis showed that the content of collagenolytic protease did not differ significantly in the latex samples, while the content of ficin decreased from L6 to L8 (supporting Fig. S1).

Total caseinolytic activity of latex samples

The changes in ficin-specific activity during fruit ripening toward casein as a substrate are presented in Fig. 2. We had already shown

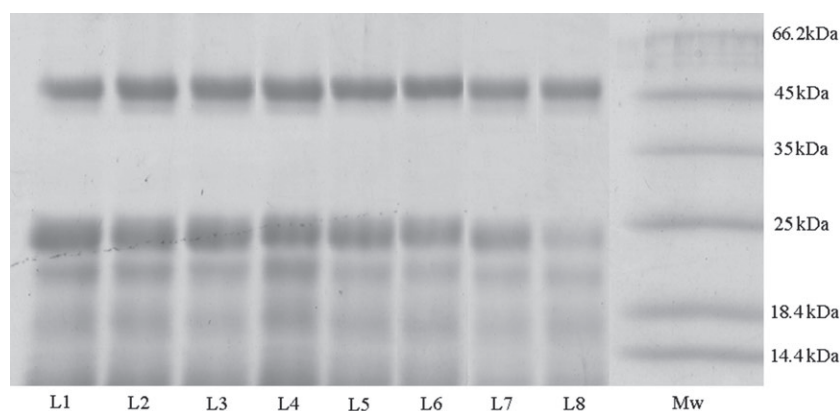


Figure 1. Electrophoretic profile of *Ficus carica* latex samples (collected between May and August: L1–L8). Mw, molecular weight markers.

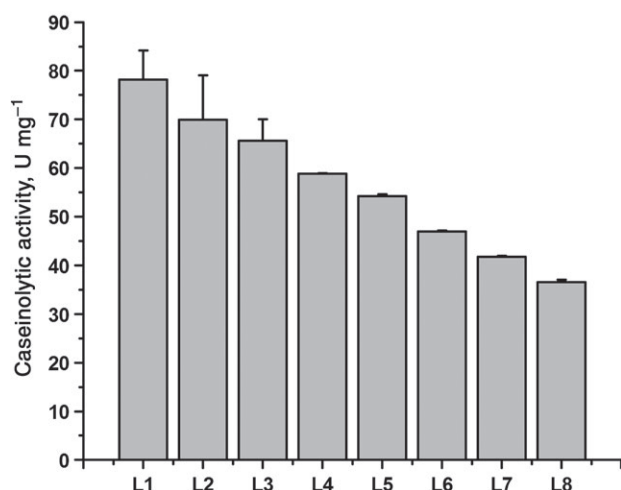


Figure 2. Caseinolytic activity of *Ficus carica* latex samples (collected between May and August: L1–L8). The values represent means \pm standard deviation of data from three independent experiments.

in our previous study that other proteolytic component of fig latex (collagenolytic serine protease) do not have specificity for casein hydrolysis,⁹ thus casein hydrolysis could be attributed to ficin only.

The results suggest that ficin specific activity decreased over time, being the highest at the beginning of fruit development (about 80 U mg⁻¹). At the moment of complete fruit ripening the activity declined to about one half of the initial activity (about 40 U mg⁻¹). However, such a decrease in specific activity of latex samples attributed to ficin could be explained by the increases in the concentrations of other proteins (Table 1), and both absolute (supporting Fig. S1) and relative (supporting Table S1) decreases of ficin band abundance. Our results confirm that ficin possesses relatively high proteolytic activity. Even the sample with the smallest proteolytic activity (L8 in Fig. 2) exhibited higher specific activity towards casein than latices of other laticiferous plants, e.g. *Ficus religiosa*, *Ficus bengalensis*, *Calatropis procera*, *Carica papaya*.²⁷

As the latex defence mechanism is assigned (to a large extent) to the proteolytic fraction, whose major constituent is ficin, it seems that fig's major defence mechanism in the early stages of fruit development relies on ficin activity. This activity is most probably replaced over time with other protection strategies, e.g. high concentrations of phenols, tannins and lignin.³

Milk clotting activity of latex samples

It has been already reported that fig latex possesses enzymes that have an appropriate milk clotting to caseinolytic activity ratio, which makes it a potential substitute for rennet in the production of cheese, since there is a constant need to identify and apply enzymes from different sources in the cheese industry due to the reduced supply of calf rennet and its high price.²⁸ Furthermore, there is on-going demand for the identification of plant-derived milk clotting proteases, as the use of animal rennet may be limited for religious reasons (e.g. in Judaism and Islam), diet (vegetarianism), or consumer concern regarding genetically engineered foods (e.g. Germany, Netherlands and France forbid the use of recombinant calf rennet).

The potential use of fig latex in the dairy industry could be of significant importance for commercial application of fig latex, which led us to explore whether there is a dependency between latex samples collected in different periods of time and milk clotting activity. The results are shown in Fig. 3A.

Figure 3A shows that there is a time-dependent increase in milk clotting activity in latex samples. The specific activity of latex samples against κ -casein was around 10 times higher in latices collected in July–August, when compared to samples collected in spring. Furthermore, when caseinolytic and milk clotting activities were compared (Fig. 2 and Fig. 3A) it appeared that summer samples are better suited for application in the cheese industry. When seeking potential plant enzyme substitutes for rennet, the most important problem is that plant proteases usually have high proteolytic activity and cause extensive digestion of casein.²⁸ As an appropriate indicator of suitability for use in the dairy industry, the ratio of milk clotting to general proteolytic activity (*C/P* value) is used,²⁹ since a higher *C/P* value means less short peptides products that contribute to bitter taste of cheese.³⁰

C/P values for fig latex samples are presented in Fig. 3B. A steeper increase can be noticed compared to milk clotting activity as a consequence of opposite trends in caseinolytic and milk clotting activity over time. The *C/P* values determined for August samples of fig latex (0.6–0.7) suggested that fig latex collected in this period could be of use in cheese making.³¹

Interestingly, we found here that while caseinolytic activity decreased, milk clotting activity increased over time and both of them could be attributed to ficin.^{17,32} However, it is known that ficin represents a heterogeneous protein fraction of fig latex, which includes at least five different isoforms.³³

In an early study by Kramer and Whitaker it was shown that different forms of ficin partially purified from *F. carica* latex had

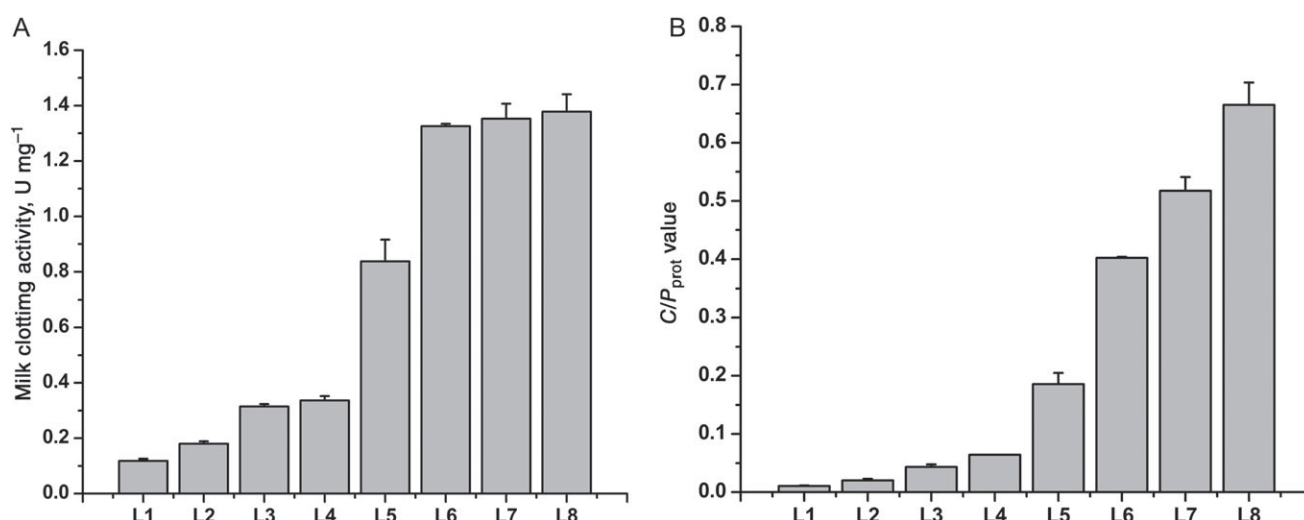


Figure 3. Milk clotting activity against milk with 5 g kg⁻¹ fat as substrate (A) and milk clotting to general proteolytic activity ratio, C/P value (B) of *Ficus carica* latex samples (collected between May and August: L1–L8). The values represent means \pm standard deviation of data from three independent experiments.

different milk clotting activities as well as total caseinolytic activities. Milk clotting activities and C/P ratios varied up to 50 times and 80 times, respectively, among different ficin isoforms.³⁴ Alteration in specificity of fig latices toward total casein (determined as caseinolytic activity) and κ -casein (determined as milk clotting activity) presented in this work could be the consequence of differential expression of ficin isoforms in fig latex during flowering and fruit ripening. At the beginning of this period, it seems that ficin forms of general caseinolytic specificity are predominantly present, enabling hydrolysis of α , β and κ -casein. As the fruit becomes more mature, it can be assumed that forms with general specificity for caseins are gradually replaced with forms that are more specific for κ -casein, giving the highest milk clotting activity per milligram of protein.

Furthermore, we examined SDS-PAGE analysis of clots and whey of milk samples coagulated with different latices obtained from the previous experiment. Results of a comparison between clot and whey content between differently coagulated milk samples are presented in Fig. 4. It can be noted that the content of milk clots differed by using latices collected in different periods (Fig. 4). In fact, the main fragment obtained after milk clotting had a molecular weight of approximately 25 kDa in all samples, but its relative amount slightly differed between the samples. The casein fragment of 25 kDa was the most abundant if mid-August fig latex was used for milk coagulation in comparison to other latices.

Fig latex collected in the period when the fruits are completely ripe could be a promising candidate as an alternative coagulating agent in the dairy industry because large volumes of fig latex could be collected at a low price.⁷ Furthermore, fig latex has been part of traditional medicine through the centuries even when taken orally,² suggesting no need for purification of coagulating enzyme from other latex ingredients. However, it is known that the use of enzymatic preparations of high caseinolytic activity could produce bitter cheese.^{30,31} There is no information in the literature regarding the optimal time for latex collection for the use in cheese production. However, our results strongly suggest that it is crucial to select the appropriate season for latex collection, because of the significant difference in the C/P ratio between the samples taken in spring, and the one taken in the middle of August. With regard to the concern that the highest C/P value is obtained for the last

latex sample, it is best to perform latex collection during summer, i.e. in July and August.

Comparison of chitinolytic activity

One of the most important physiological roles of fig latex includes protection from herbivorous insects via chitin degradation. It has been already suggested that chitinases and chitinase-like proteins are abundantly accumulated in plant latex, and that their existence is crucial in defence against insects. One possible defence mechanism could include chitin hydrolysis, as chitin represents the main constituent of insect shells.³⁵ This led us to also characterise fig latices in the terms of chitinolytic activity.

Changes in specific chitinolytic activity of latex samples during time are presented in Fig. 5. It can be seen that quite a regular trend exists, and that there was a gradual decrease of specific activity towards chitin. The decrease was much more intense compared to proteolytic activity. The level of specific chitinolytic activity at the beginning of fig plant flowering was almost 6.5 times higher than the activity in the period when fruits were completely ripe. The time dependent loss of chitinolytic activity suggests that the mechanism of protection of *F. carica*, which relies on chitin hydrolysis, is probably most important in the early phases of fruit development.

Kim *et al.* reported that the fruit hormone jasmonic acid is a negative effector of expression of the chitinase gene in *F. carica* latex.³⁶ It is also known that jasmonic acid is present in fruit in low concentration in the late phases of ripening. In fact, there are numerous reports that have shown that exogenously applied jasmonic acid delays ripening.^{37,38} Moreover, there is evidence that jasmonic acid has a positive effect on expression of defence-related genes.³⁹ It can be concluded, taking our results into account, that the chitinase dependent protection mechanism reduces over time.

Antifungal activity

Many papers dealing with the antimicrobial activity of *F. carica* latex have been published. Most consider organic solvent extracts, e.g. methanol, hexanol, ethyl acetate extracts, which are shown to possess antibacterial and antifungal activities. These

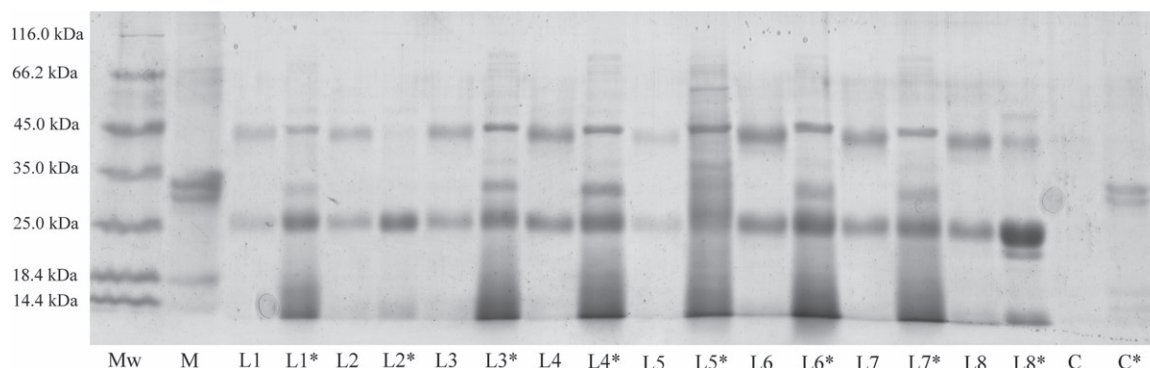


Figure 4. SDS-PAGE profiles of whey (L1–L8) and clots (L1*–L8*) of *Ficus carica* latex samples collected between May and August against milk (M) with 5 g kg⁻¹ fat as a substrate. Mw, molecular weight markers; C, chymosin whey; C*, chymosin clot.

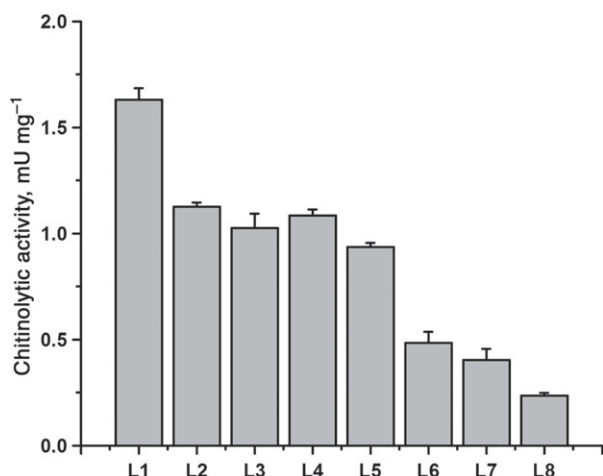


Figure 5. Chitinolytic activity of *Ficus carica* latex samples (collected between May and August: L1–L8). The values represent means \pm standard deviation of data from three independent experiments.

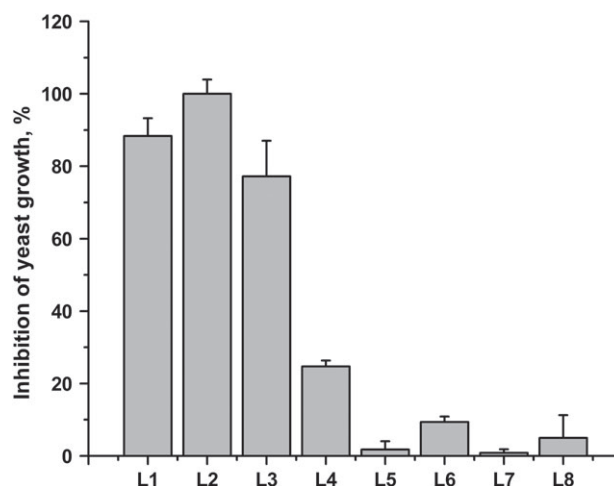


Figure 6. Antifungal activity of *Ficus carica* latex samples (collected between May and August: L1–L8) against *Saccharomyces cerevisiae*. The values represent means \pm standard deviation of data from three independent experiments.

activities are assigned to low molecular weight compounds of fig latex.^{40,41}

Although interest in the investigation of antifungal activity of *F. carica* latex has been present for many years, antifungal activity of the aqueous phase of fig latex is not yet well understood. A modest number of studies have revealed the existence of antifungal proteins in the aqueous phase of latex of genus *Ficus*. However, few new antifungal proteins have been discovered recently. Some of them include the chitinase of *Ficus awkeotsang*, as well as two chitinases isolated from the latex of *Ficus microcarpa*.⁴²

Results on the antifungal activity of *F. carica* latex against *S. cerevisiae* are presented in Fig. 6. It can be noted that there was a steep decline in antifungal activity from May to August, and that antifungal activity was most pronounced in spring, more precisely in May. Such a result is not surprising since it is in accord with our result of chitinolytic activity. In fact, the great majority of recently described antifungal proteins of genus *Ficus* are chitinases, or chitinase-like enzymes.^{13,14}

Identification of the active compounds of *F. carica* latex and improved understanding of the mechanism of action against fungus-induced diseases of crops may result in substitution of previously applied phytotoxic and environmentally harmful substances for non-toxic ones such as latex. The potential for utilising this natural extract in organic agriculture should be investigated further.

Moreover, another interesting potential application of *F. carica* latex could be as a natural food preservative in the food processing industry, especially when we consider that fig latex has a high content of minerals, vitamins, antioxidants, fibre and proteins, which makes it a valuable functional food.⁴³

CONCLUSIONS

The overall protein concentration, ficin activity and specificity, as well as chitinase and antifungal activities differed in fig latices collected from the beginning of flowering until complete fruit ripening. The protection mechanism against insects and fungi relies on chitinolytic activity, which is most important in the early phases of flowering and is replaced with other protection strategies over time. Forms of ficin with different casein specificities are present in different proportions during fruit ripening, which is of importance for applications in the dairy industry.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Identification, purification and characterization of a novel collagenolytic serine protease from fig (*Ficus carica* var. Brown Turkey) latex

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A novel collagenolytic serine protease was identified and then purified (along with ficin) to apparent homogeneity from the latex of fig (*Ficus carica*, var. Brown Turkey) by two step chromatographic procedure using gel and covalent chromatography. The enzyme is a monomeric protein of molecular mass of 41 ± 9 kDa as estimated by analytical gel filtration chromatography. It is an acidic protein with a pI value of approximately 5 and optimal activity at pH 8.0–8.5 and temperature 60°C. The enzymatic activity was strongly inhibited by PMSF and Pefabloc SC, indicating that the enzyme is a serine protease. The enzyme showed specificity towards gelatin and collagen (215 GDU/mg and 24.8 CDU/mg, respectively) and non-specific protease activity (0.18 U/mg against casein). The enzyme was stable and retained full activity over a broad range of pH and temperature. The fig latex collagenolytic protease is potentially useful as a non-microbial enzyme with collagenolytic activity for various applications in the fields of biochemistry, biotechnology and medicine.

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[Key words: Collagenolytic activity; Gelatinolytic activity; Serine protease; Fig latex; Enzyme purification]

Collagenases are enzymes that cleave the polypeptide backbone of native collagen. These proteases comprise two major groups: metallo-collagenases and serine-collagenases. Metallo-collagenases are zinc-containing enzymes that usually also require calcium for their optimum activity and stability. These enzymes have been widely studied from various mammalian tissues (where they play a role in remodeling of extracellular matrix) (1) as well as from bacteria, principally from *Clostridium histolyticum* (2).

Serine collagenolytic proteases (serine collagenases) were found in many species of crab, other crustaceans and fish in which they are involved in food digestion (3). In variance to mammalian and bacterial metallo-collagenases which exhibit exclusive specificity for collagen, serine collagenases reported to date possess broad proteolytic activities in addition to collagenolytic activity (4,5).

Collagenases have a number of industrial, biotechnological, pharmacological and medicinal applications. Collagenases are used as meat tenderizers in food industry (6) and have applications in fur and hide tanning to improve the exhaustion of dye (7). Collagenases are applied for the isolation and cultivation of the mammalian cells in culture (8) and cleaning blood for improved screening in medical diagnostics (9). They are used to treat burns and ulcers (10,11), to eliminate scar tissue (12,13) and play an important role in the successful transplantation of specific organs (14,15). Therapeutic applications include wound healing (10), treatment of Peyronie's disease (16), and various types of destructive fibrosis, such as liver cirrhosis (17). Peptides that are formed upon enzymatic hydrolysis of collagen, as well as of gelatin find wide applications, e.g.,

cosmetics moisturizers, dietary materials, immunotherapeutic agents, agents for treating osteoporosis, gastric ulceration, and hypertension (18).

Given the potential uses of collagenases and their high demand, there is an interest in finding new sources of collagenolytic enzymes with novel properties. Plant collagenases have been less studied compared to those of animal and bacterial origin. The only collagenase characterized to date is ginger collagenolytic cysteine protease, a papain-like enzyme with broad substrate specificity (19). The latex of the common fig (*Ficus carica*) is known as a rich source of proteolytic activity which is attributed principally to ficin, a potent cysteine protease with broad substrate specificity (20). In the course of studying ficin from fig (*F. carica* var. Brown Turkey) latex, we observed that it contains, in addition to ficin, a novel protease with gelatinolytic activity, which has passed unnoticed so far. This prompted us to initiate the present study with the aim to identify, purify and characterize this activity. We showed that novel protease is highly pH and temperature stable collagenolytic serine protease with high specificity towards gelatin and collagen and with very low non-specific protease activity. These, together with easy availability of fig latex, make large-scale production of the enzyme possible and thereby enable investigation of its biomedical and biotechnological applications.

MATERIALS AND METHODS

Materials Casein, collagen from rat tail Na-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), iodoacetamide (IAA), *N*-[N-(1-3-trans-carboxyirane-2-carbonyl)-L-leucyl]-agmatin (E-64), phenylmethanesulfonyl fluoride (PMSF), pepstatin A, Pefabloc SC, benzamidin hydrochloride, soybean trypsin inhibitor (STI), bovine serum albumin (BSA), carbonic anhydrase from bovine erythrocytes, cytochrome c from equine heart, polyethylene glycol (PEG-6000), bromophenol blue

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(BPB) and L-cysteine were purchased from Sigma–Aldrich (Steinheim, Germany) and gelatin (from bovine skin) was purchased from Merck (Darmstadt, Germany). Unstained protein molecular weight markers were from Thermo Fisher Scientific (Rockford, IL, USA). Thiol-Sepharose was from GE Healthcare (Uppsala, Sweden). All other chemicals were of analytical grade and were used without further purification.

Collection of latex Fresh latex was collected from the fig (*F. carica* var. Brown Turkey) trees growing in the private orchard in Bar, Montenegro, by separation of immature green fruits from tree shoots. All latex samples used here were collected in the middle of August. The latex fluid was poured into 1.5 mL micro-centrifuge tubes and was immediately stored at -20°C until used.

Preparation of latex water fraction The frozen latex was thawed at 4°C and was centrifuged at $10,000 \times g$ at 4°C for 60 min to remove insoluble gum (approximately 20% of weight) and other debris. The supernatant was then subjected to extraction with two volumes of petroleum ether to completely eliminate polymeric gum. This process was repeated three times (21). The clear water fraction is referred to as latex water fraction (LWF). Aliquots of 1 mL of LWF were frozen in liquid nitrogen and stored at -20°C until used.

Protein concentration was determined by the biuret method (22). Protein concentration in LWF varied depending on the latex batch, from 81 ± 9 mg/mL to 106 ± 8 mg/mL. We opted for biuret method because it gave values for LWF protein concentration comparable to those reported previously by applying the same method (23). In our hands, Bradford method, which is routinely used at present for protein concentration determination in biological material including major fig latex fraction ficin (20), gave highly underestimated values for LWF protein concentration compared to those obtained by the biuret method. It is of note that protein concentration in commercial preparations of ficin (e.g., Sigma–Aldrich) is also determined by applying biuret method.

Purification of collagenolytic fraction Aliquots of LWF were thawed and then pre-incubated at room temperature for 1 h with cysteine (final concentration 52 mM) to activate ficin (24). The reaction mixture was then applied to Sephadex G-50 column (1×30 cm). The fractions containing high molecular weight proteins with both caseinolytic and gelatinolytic activity were pooled and concentrated by ultrafiltration. The pooled and concentrated protein fractions were then applied on to a thiol-Sepharose column (1×10 cm) which was pre-equilibrated with 0.1 M Tris–HCl buffer pH 8.0 (buffer A). Unbound proteins were eluted from the column with buffer A ($A_{280} \text{ nm} < 0.05$) followed by the elution of covalently bound fraction of ficin by buffer B (buffer A containing 20 mM L-cysteine) (25). Fractions were collected at the flow of 0.8 mL/min. Gelatinolytic activity in fractions of unbound proteins was detected by gelatin SDS-PAGE. The fractions containing enzymatic activity were pooled, concentrated by ultrafiltration and then used for characterization.

Electrophoresis, protein staining and gelatinolytic activity staining SDS-PAGE in 12% resolving gel and 4% stacking gel was performed in protein electrophoresis unit GV202 (Bio-Step, Jahnndorf, Germany) under non-reducing conditions according to Laemmli (26). Before loading on to the gel, the LWF samples were pre-treated with L-cysteine as described above. The gels were stained with Coomassie Brilliant Blue (CBB) as described in Bollag et al. (27). The molecular mass of the protein band was estimated by comparison with standard molecular weight markers (14.4–116 kDa). Calibration curve was created by plotting mobility of bands of standard molecular weight markers against logarithm of their molecular weights.

Two kinds of gelatin PAGE were carried out: gelatin SDS-PAGE and 2D gelatin PAGE as described by Gavrovic-Jankulovic et al. (28) and Bollag et al. (27), respectively. After electrophoresis, the gels were washed three times with TBS buffer (Tris-buffered saline, 50 mM Tris containing 0.15 M NaCl and 7 mM Na-azide, pH 8.1) containing 10 mM L-cysteine prior to incubation in the same buffer for 16 h at room temperature under gentle shaking in the dark (28). Following incubation, the gels were stained with CBB. The gelatinolytic activities were identified as clear zones on blue background on the gel. The intensity of the bands was quantified using the Gel-Pro 3.1 software (Media Cybernetics) with the single band analysis option. 2D gelatin PAGE comprised IEF in the presence of 8 M urea (27) followed by gelatin SDS-PAGE in the second dimension as described above. After the focusing, the pH gradient was determined by cutting the empty gel stripe into 0.5 cm slices. Each slice was incubated in 10 mM KCl for 30 min and pH value of KCl solutions was determined and plotted against the distance of the gel slice from the acidic electrode. pI value of collagenolytic protease was calculated from the plot as previously described (27).

Gel filtration analysis of purified collagenolytic protease Analytical gel filtration of purified collagenolytic protease was performed in PBS using Äkta Purifier 10 System (GE Healthcare, Uppsala, Sweden) on Superdex 75 PC 3.2/30 column according to manufacturer's instructions. The calibration of the column was performed using the mixture of gel filtration molecular weight standards (1 mg/mL each): bovine serum albumin, carbonic anhydrase and cytochrome c.

Enzyme assays Enzymatic activity of LWF and purified enzyme fractions were assayed using the following substrates: casein (non-specific protease substrate) and BAPNA, both of which were applied as substrates in enzymatic assay of ficin, gelatin (substrate of both collagenase and gelatinase) and native collagen (substrate of collagenase).

Caseinolytic activity assay Casein hydrolysis was performed essentially as described by Devaraj et al. (20). Briefly, samples were incubated with a 0.5 mL of 0.65% casein solution in TBS containing 10 mM L-cysteine. After 30 min incubation at 37°C , 1 mL of 5% (w/v) TCA (trichloroacetic acid) was added to terminate the reaction followed by the centrifugation at $12,000 \times g$ for 15 min. The absorbance of TCA-soluble products was measured at 280 nm. The assay included appropriate blanks. One arbitrary unit (U) of caseinolytic activity was defined as the amount of enzyme that gives rise to an increase in absorbance at 280 nm per minute of digestion under optimum conditions of the assay. Number of arbitrary units of activity per milligram of protein (U/mg) was taken as specific activity of enzyme.

Hydrolysis of BAPNA Hydrolysis of BAPNA was quantified as described by Devaraj et al. (20) with minor modifications. Briefly, BAPNA, dissolved in DMSO (dimethyl sulfoxide), was diluted with TBS containing 10 mM L-cysteine to a final concentrations of 2 mM BAPNA and 5% of DMSO. Assay was performed by incubating samples with 120 μL of freshly prepared BAPNA solution in micro-plate well (Sarstedt, Newton, NC, USA). After 60 min incubation at room temperature (22°C), 100 μL of 50% acetic acid was added to terminate the reaction and absorbance at 405 nm was recorded in LKB Micro plate reader 5060-006 (GDV, Roma, Italy) against the blank. One unit of enzyme activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of BAPNA per min (the extinction coefficient of p-nitroaniline: $\epsilon = 8800 \text{ cm}^{-1} \text{ M}^{-1}$ at 405 nm). The specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U/mg.

Collagenolytic and gelatinolytic activity assays The enzymatic activity towards both collagen and gelatin was measured using the ninhydrin method as described by Zhang et al. (29) with minor modifications. Briefly, collagen or gelatin (at concentrations of 5 mg/mL and 20 mg/mL respectively) were slowly dissolved in 50 mM Tris–HCl buffer pH 8.1 at 37°C for 15 min. A reaction mixture containing 100 μL of collagen or gelatin solution and 10 μL of sample solution was incubated at 37°C for 5 h with gentle shaking. The reaction mixture was incubated for 1 h at 4°C after addition of 110 μL of 20% (w/v) PEG 6000 solution. The solution was centrifuged at $12,000 \times g$ for 30 min at 4°C . The assay included appropriate blanks. The supernatant (20 μL) was mixed with 200 μL of ninhydrin solution, incubated at 100°C for 10 min, then cooled to room temperature. Subsequently, the mixture was diluted with 400 μL of 50% 1-propanol. Absorbance at 570 nm was recorded against the blank containing only ninhydrin but without substrate and sample. One arbitrary unit of collagen or gelatin digestion activity (CDU and GDU, respectively) was defined as the amount of enzyme that releases peptides from collagen or gelatin equivalent in ninhydrin color to 1 μmol of leucine in 5 h. The specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U/mg.

Effect of inhibitors and metal ions on enzymatic activity The effect of the following inhibitors on proteolytic, gelatinolytic and collagenolytic activity of LWF and gelatinolytic activity of purified collagenolytic fraction was tested: IAA (cysteine protease inhibitor), PMSF (serine protease inhibitor), EDTA (metalloprotease inhibitor), at the concentration of 2 mM; E-64 (cysteine protease inhibitor) at the concentration of 22.5 μM and pepstatin A (aspartic protease inhibitor) at the concentration of 14.6 μM . The aliquots of sample solutions diluted with TBS were pre-incubated with the inhibitors for 30 min at room temperature (22°C) prior to the assay of the enzymatic activity. The residual activity was determined as the percentage of the proteolytic activity in an inhibitor-free control sample.

Effect of inhibitors and metal ions (Ca^{2+} and Zn^{2+}) on purified collagenolytic fraction was examined by gelatin SDS-PAGE. Briefly, after electrophoresis and washing of gels, replicate gels were sliced (in triplicate) and each slice was then incubated (for 16 h) in the buffer in which one of the inhibitors (at above concentrations) or metal ions (2 mM) were added. Following incubation, the gels were stained with CBB. The inhibition was expressed as a percentage of intensity of the proteolytic bands in the presence of inhibitor compared to a control.

Collagen cleavage assay Native collagen cleavage assay was performed as described by Kim et al. (19) with minor modifications. The reaction mixtures contained 15 μL of 0.1 mg/mL collagenolytic fraction and 60 μL of 0.5 mg/mL collagen in PBS. After 16 h incubation at 37°C , the reaction was prepared for SDS-PAGE analysis by addition of sample buffer and boiling for 5 min at 95°C . Reaction mixtures in which the enzyme was pre-incubated with 2 mM PMSF, 0.1 mM Pefabloc SC, 2 mM benzamidinium hydrochloride and 1 mM STI were also assayed. The comparison of influence of four different serine protease inhibitors was observed by analysis of collagen degradation products on 7.5% SDS-PAGE gel.

Effect of pH and temperature on enzyme activity and stability To determine the optimum pH for enzyme activity, pH of the reaction mixture containing 2% (w/v) gelatin was varied over the range 3.0–9.5, while for the pH stability tests, the enzyme was pre-incubated for 30 min at the selected pH (from pH 3.0 to 9.5), and then the enzymatic activity was determined at pH 8.0. The following buffers were used: 50 mM Na citrate buffer (pH 3.0–6.0), 50 mM Na phosphate buffer (pH 6.5 and 7.0), 50 mM Tris–HCl buffer (pH 8.0 and 9.0) and 50 mM Gly buffer (pH 9.5). To determine the optimum temperature for enzyme activity, the reaction mixture was incubated at the selected temperature (from 20°C to 80°C), while for the thermostability tests the enzyme was pre-incubated for 30 min at the selected temperature (from 20°C to 90°C), and then the enzymatic activity was determined at 37°C . The residual activity was calculated as the ratio between the enzymatic activity,

observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%) of maximal activity.

RESULTS AND DISCUSSION

Identification and quantification of collagenolytic activity in fig latex We first set to characterize crude latex with respect to its gelatinolytic activity, to establish whether it is associated with collagenolytic activity and if so to characterize it more closely in order to facilitate its isolation and further examination. Measurement of fig latex gelatinolytic, collagenolytic and proteolytic activity is presented in Table S1. All measurements were done using fig latex water fraction (LWF) which represents latex devoid of gum and other debris. Table S1 shows that LWF exhibited substantial gelatinolytic activity (62 ± 2 GDU/mg) which was associated with collagenolytic activity (6.4 ± 0.1 CDU). The caseinolytic activity of LWF (1.1 ± 0.2 U/mg) agrees well with that reported previously for the caseinolytic activity of crude fig latex (30), which was attributed to ficin in the cited study. The low (0.040 ± 0.001 U/mg) hydrolyzing activity of LWF towards BAPNA may be explained by the low activity of ficin towards this substrate (20). Values are expressed as a mean \pm standard deviation. Each value is the average of the results of three experiments.

SDS-PAGE of LWF revealed major bands at around 48 kDa and 25 kDa as well as several protein/peptide bands of molecular mass below 14.4 kDa (Fig. 1A). Gelatinolytic band appeared only at a position of approximately 48 kDa on SDS-PAGE (Fig. 1B) suggesting that this protein represents fig latex gelatinolytic/collagenolytic activity. It is of note that the protein profile and LWF activity towards gelatin remained unchanged for the latex batches collected in 3 consecutive years and kept frozen at -20°C (Fig. 1). The protein of 25 kDa from Fig. 1A was assigned to ficin since its molecular mass was close to that recently determined by mass spectrometry for pure ficin ($23,500 \pm 500$ Da) (20). The LWF protein profile from Fig. 1A agrees with SDS-PAGE of *F. carica* latex proteins reported in a recent study (31).

Relative activity of LWF enzymes in the presence of five inhibitors was investigated in order to establish the enzymatic class of gelatinolytic/collagenolytic activity. Fig. 2 shows that both gelatinolytic and collagenolytic activity of LWF were inhibited completely by PMSF, but not by any of the other protease inhibitors.

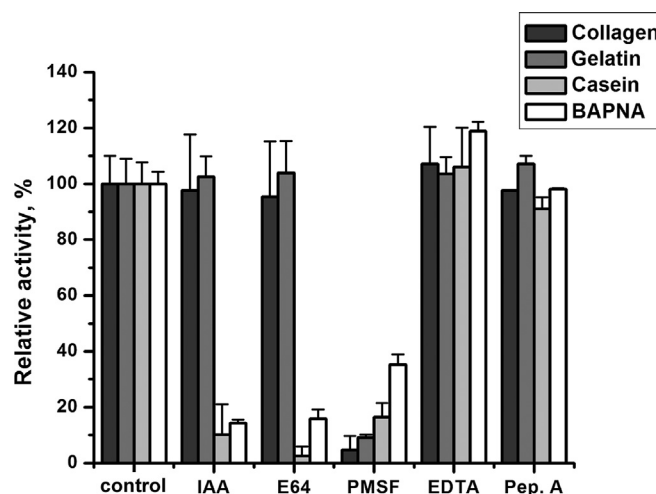


FIG. 2. Effects of inhibitors on LWF collagenolytic and proteolytic activities. The aliquots of LWF diluted with PBS were pre-incubated with the selected inhibitor for 30 min at room temperature (22°C) prior to the assay of the enzymatic activity. The residual activity was determined as the percentage of the enzymatic activity in an inhibitor-free control sample. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

These preliminary results suggest that fig latex contains novel enzyme which is a collagenolytic serine protease since the enzyme hydrolyzed collagenous substrates and was inhibited by the irreversible inhibitor of serine proteases. Caseinolytic activity of LWF was nearly completely inhibited by cysteine protease inhibitors (IAA and E-64) and partially by PMSF suggesting that this activity was associated essentially with ficin. In the presence of EDTA activity of both, ficin and collagenolytic serine protease slightly increased (Fig. 2). Increase in activity of ficin is explained by the elimination of trace amounts of heavy metal ions from solution by EDTA (32). Investigation of the effect of EDTA on collagenolytic activity is described below.

Purification and characterization of collagenolytic serine protease

Based on above described results obtained with crude latex we devised a two step chromatographic procedure which yielded both, novel collagenolytic protease and ficin purified to homogeneity. The procedure included gel chromatography of LWF (figure not shown) followed by covalent chromatography of the first fraction eluted from Sephadex column on thiol-Sepharose column and analytical gel filtration chromatography of purified collagenolytic protease (Fig. 3). The collagenolytic protease was purified 3.9 fold with 94% yield and a specific activity of 24.8 CDU/mg (on collagen) and 215 GDU/mg (on gelatin). Ficin was purified 2.5 fold with 75% yield and a specific activity of 2.7 ± 0.3 U/mg. The procedure is simple and highly reproducible with respect to yields of both enzymes within experimental error. These, together with easy availability of fig latex, make large-scale production of collagenolytic protease possible and thereby enable investigation of its structure function relationship as well as of its biomedical and biotechnological applications. The details of purification are summarized in Table S2. The single peak of around 41 kDa was obtained as a result of the analytical gel filtration chromatography (Fig. 3C). The purified enzyme showed a single band of 48 ± 2 kDa on SDS-PAGE, under both non-reducing (Fig. 4A) and reducing conditions (not shown) as well as on gelatin SDS-PAGE (Fig. 4B), which, taken together with the result of gel filtration, suggest the monomeric nature of the enzyme. The single band on gelatin 2D PAGE (Fig. 4C) with isoelectric point of pH 5 indicates that the protein is an acidic protein.

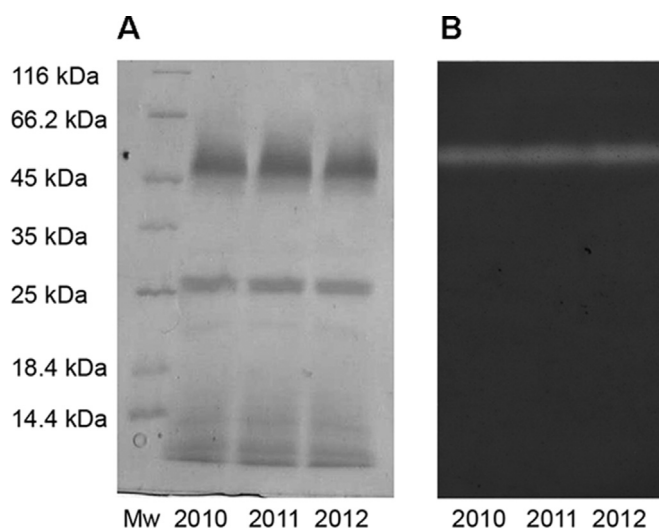


FIG. 1. SDS-PAGE (A) and gelatin SDS-PAGE (B) of LWF proteins. Mw, molecular mass standards. Samples of LWF from latices collected in three consecutive years (2010 – 2012 from figure), and kept frozen at -20°C are shown.

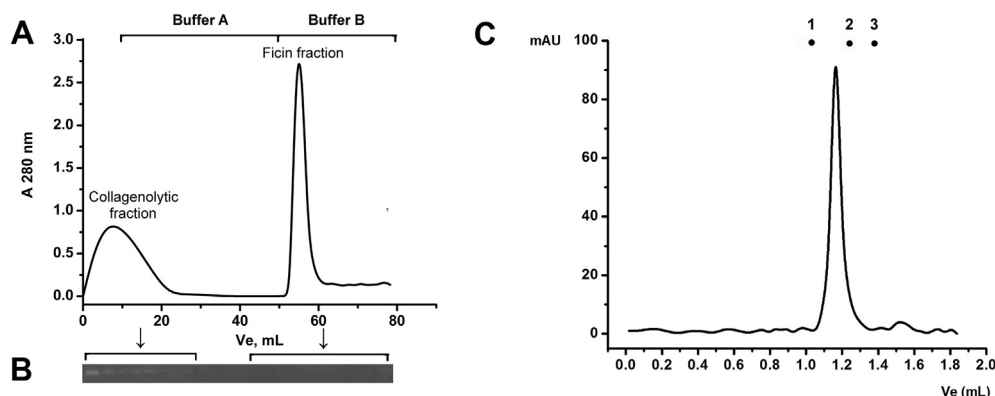


FIG. 3. (A) Chromatographic separation of the first fraction eluted from Sephadex G-50 column on thiol-Sepharose column. Column was equilibrated and eluted with 0.1 M Tris-HCl buffer pH 8.0 (buffer A) followed with elution with the same buffer containing 20 mM cysteine (buffer B). The fractions were collected with a flow rate of 0.8 mL/min. (B) Activity of each fraction towards gelatin was determined by gelatin SDS-PAGE. (C) Gel filtration analysis of purified collagenolytic protease. Points 1, 2, and 3, gel filtration molecular weight standards (bovine serum albumin, carbonic anhydrase and cytochrome c, respectively).

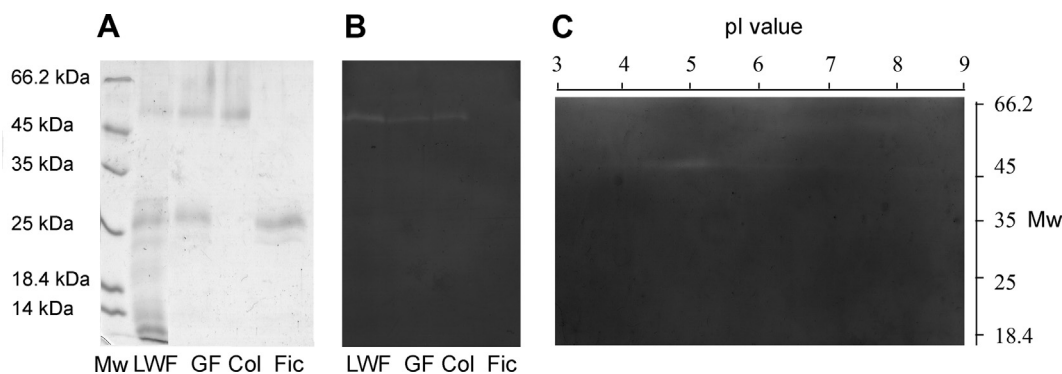


FIG. 4. Electrophoretic analysis of LWF proteins during purification: (A) SDS-PAGE; (B) gelatin SDS-PAGE; (C) gelatin 2D PAGE of purified collagenolytic protease. GF, first fraction eluted from Sephadex G-50 column, Col, collagenolytic serine protease, Fic, ficin separated from GF by means of thiol-Sepharose column.

The enzyme exhibited very low non-specific protease activity. Thus, its hydrolyzing activity against casein was 0.18 ± 0.02 U/mg and activity against BAPNA was undetectable. In that respect, fig latex collagenolytic protease is comparable to the preparations of collagenase from *C. histolyticum* (33) which find wide applications in the field of biotechnology and medicine (7,11–15,34).

Native collagen cleavage and the effect of inhibitors and metal ions on collagenolytic activity

Our results clearly

indicate that novel fig latex collagenolytic protease can cleave both chains of native type I collagen molecule ($\alpha 1$ and $\alpha 2$), as well as β and γ components (Fig. 5B).

PMSF inhibited more than 90% of the collagenolytic/gelatinolytic activity of the purified enzyme (Fig. 5A) confirming that this enzyme belongs to the serine protease class. In a more detailed study, inhibition experiments were set with three additional serine protease inhibitors (Fig. 5B). Highly specific serine protease inhibitor Pefabloc SC, as well as PMSF, inhibited collagenolytic activity,

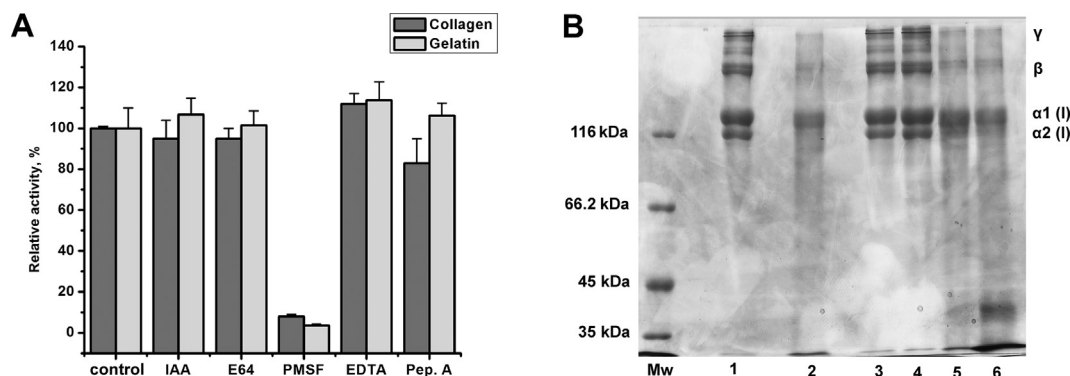


FIG. 5. Effect of inhibitors on activity of purified fig latex collagenolytic protease estimated by collagen/gelatin hydrolysis. (A) Histogram showing a percentage of intensity of the proteolytic bands in the presence of inhibitor compared to a control (collagenolytic serine protease (Col)). Each value is the average of the results of three experiments, and the error bars show the standard deviations. (B) Degradation of collagen type I by fig latex collagenolytic protease. Lane 1, collagen control; lane 2, non-inhibited collagenolytic protease; lanes 3, 4, 5 and 6, PMSF, Pefabloc SC, benzamidine hydrochloride and STI inhibited collagenolytic protease, respectively.

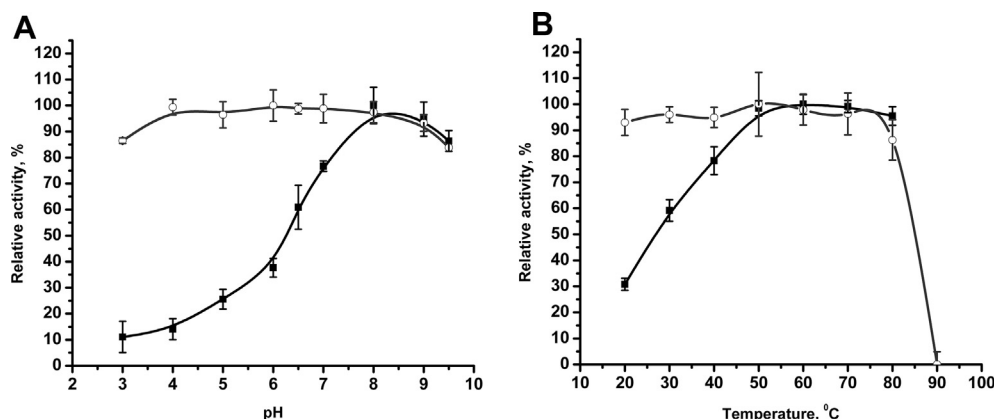


FIG. 6. Effect of pH (A) and temperature (B) on the activity (closed squares) and stability (open circles) of purified collagenolytic protease (expressed relative to maximal activity). For pH stability, the enzyme was incubated for 30 min at 22°C at the required pH, and the residual activity was measured at 37°C and pH 8.0. For temperature stability, the enzyme was incubated at the required temperature for 30 min, and the residual activity was measured at 37°C and pH 8.0. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

while benzamidine and soybean trypsin inhibitor did not show any inhibitory effect (Fig. 5B). These, taken together with undetectable hydrolysis of BAPNA indicate that the purified enzyme is serine protease with specificity different than trypsin. EDTA increased (c.a. 20%) the enzymatic activity of collagenolytic serine protease (Fig. 5A). Previous study demonstrated that zinc is an effective inhibitor of some serine collagenases (35) which prompted investigation of the effect of Zn(II) ions on fig latex collagenolytic activity. We found that Zn(II) at 2 mM concentration inhibited the enzyme activity for approximately 20%, which is significantly lower than found in the cited study (c.a. 80%). Calcium ions did not show any considerable effect on the collagenolytic activity up to 2 mM.

Effect of pH and temperature on enzyme activity and stability The effect of pH and temperature on enzyme activity and stability is presented in Fig. 6. The enzyme showed maximum activity at pH 8.0–8.5 and temperature of 60°C (Fig. 6A and B, respectively). Enzyme retained more than 80% of the activity when pre-incubated at the range of pH 4–9 and temperature 20–80°C (Fig. 6A and B, respectively). The high stability of the enzyme against pH and temperature together with its selectivity for gelatin makes this enzyme potentially beneficial in industrial applications such as tenderization of meat with high connective tissue content during cooking.

Two serine proteases from latices from plants of genus *Ficus* have been described recently: religiosin from *Ficus religiosa* (36) and benghalensin from *Ficus benghalensis* (37). Importantly, novel fig latex collagenolytic protease resembles these two enzymes with respect to molecular mass, pI, optimal pH and temperature as well as with respect to high stability against pH and temperature, but not with respect to substrate specificity. While, as described above, fig latex collagenolytic protease exhibited specificity towards collagenous substrates and low caseinolytic activity, both enzymes from cited studies exhibited essentially higher caseinolytic activity (c.a. 60 U/mg) and religiosin in addition exhibited gelatinolytic activity upon gelatin SDS-PAGE. Together the results may suggest that fig latex collagenolytic serine protease described in this work is a member of larger family of serine proteases from latices/plants from genus *Ficus* which share similar physical properties and structural characteristics but differ in substrate specificity.

Physiological role of fig latex collagenolytic protease needs to be elucidated. The presence of this enzyme along with ficin in fig latex may explain its effectiveness in treatments of warts, boils, scars and dermatitis for which it has been traditionally used in folk medicine worldwide (38). Latices from several plants of genus *Ficus* including

F. carica and *F. religiosa* as well as from many other laticiferous plants were reported to show gelatinolytic activity (39) which points to latex as a potential source of collagenolytic enzymes which is understudied at present.

Conclusion In the current study novel collagenolytic serine protease was identified and then purified (along with ficin) to apparent homogeneity from the latex of fig (*F. carica*, var. Brown Turkey). The purified enzyme is a monomeric protein with molecular mass of 41 ± 9 kDa estimated by analytical gel filtration chromatography and pI value of approximately 5. The specificity and activity towards collagenous substrates (both gelatin and collagen), as well as high stability with respect to pH and temperature makes this enzyme potentially useful in various applications in the fields of biotechnology and medicine.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jbiosc.2014.05.020>.

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Table S1. Gelatinolytic, collagenolytic and proteolytic activity of LWF.

Substrate	Specific activity
Gelatin	62 ± 2 GDU/mg
Collagen	6.4 ± 0.1 CDU/mg
Casein	1.1 ± 0.2 U/mg
BAPNA	0.040 ± 0.001 U/mg

Table S2. Summary of purification of collagenolytic serine protease from LWF.

Steps	Total Protein (mg)	Total Activity (CDU)	Specific Activity (CDU/mg) ^a	Activity Yield (%)	Purification Fold
LWF	80	528 ± 8	6.4 ± 0.1	100	1
Sephadex G-50	47	509 ± 8	10.8 ± 0.3	96	1.7
Thiol-Sepharose	20	496 ± 4	24.8 ± 0.2^b	94	3.9

^aEnzymatic activity was measured on collagen. ^bSpecific activity of purified collagenolytic serine protease on gelatin was 215 ± 5 GDU/mg.



Evidence of β -sheet structure induced kinetic stability of papain upon thermal and sodium dodecyl sulfate denaturation

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Abstract: Papain is a protease that consists of α -helical and β -sheet domains that unfold almost independently. Both, considerable thermal stability and sodium dodecyl sulfate (SDS) resistance of papain have been shown. However, the ability of each domain to unfold upon thermal and SDS denaturation has never been studied. This work shows that fruit papain has slightly higher resistance to thermal inactivation when compared to that of stem papain with a rather high activation energy (E_a) of 223 ± 16 kJ mol⁻¹ and a T_m 50 value of 79 ± 2 °C. The SDS resistance of fruit papain was estimated by SDS–PAGE analysis and activity staining. It was noted that, in the presence of SDS the protein remained active, unless heat energy was applied in order to unfold papain. Furthermore, it was proven *via* Fourier transform infrared spectroscopy (FT-IR) that an α -helical domain of fruit papain is more prone to unfolding at elevated temperatures and in the presence of SDS than a β -sheet rich domain. Thermal denaturation of papain without detergent present led to accelerated formation of aggregation specific intermolecular β -sheets as compared to native protein. The presented results are of both fundamental and practical importance.

Keywords: thermal inactivation; SDS resistance; secondary structure; FT-IR spectroscopy.

INTRODUCTION

Papain is a cysteine protease (EC 3.4.22.2) with broad substrate specificity and numerous applications. The preprotein consists of 345 amino acids and it is secreted as zymogen.¹ After cleavage of the activation peptide, the mature enzyme contains 212 amino acid residues that are organized in two domains. The *N*-terminal domain has mainly α -helical structure, while the C-terminal domain

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has an antiparallel β -sheet fold.² Commercial papain can be isolated from latex (stem) or papaya fruit. Both commercial enzymatic preparations have broad substrate specificity.³

Papain is extensively used as meat tenderizer,⁴ in dental caries removal procedures,⁵ for preparation of clinically relevant antibody fragments,⁶ as a cell dissociation/debris removal agent⁷ and as a component in cosmetic preparations.⁸ Papain has been used in the detergent industry since the enzyme shows high activity and broad specificity for hydrolysis of peptide bonds.⁹ Furthermore, the enzymes from the papain family are known to be sodium dodecyl sulfate (SDS) resistant¹⁰ and very stable enzymes at elevated temperatures.¹¹

Elucidation of the basis for the extreme temperature and SDS stability of proteins is both of fundamental and practical importance. Recent studies suggested that a rigid β -sheet fold, which leads to low structural flexibility of a protein, could be responsible for the SDS and protease resistance and might also be responsible for thermal stability of a protein.¹⁰ Since papain has both α -helical and β -sheet domains, the aim of this study was to check if any of the domains was more prone to SDS denaturation by monitoring the changes in the secondary structures of the protein at elevated temperatures and in the presence of SDS. The kinetic parameters of the thermal inactivation of fruit papain were also estimated.

EXPERIMENTAL

Papain purification

Fruit papain was purified from a commercial papain preparation (BDH, London, England). Papain was extracted from the dry powder with 100 mM Tris buffer pH 8 containing 100 mM NaCl and 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA) for 30 min at 10 °C. The extract was centrifuged for 15 min at 4000×g at 10 °C and the obtained supernatant was further used. Papain was precipitated from supernatant by addition of 2 volumes of ice-cold acetone followed by incubation at –20 °C for 30 min. The precipitate was separated by centrifugation at 10,000×g for 5 min. at 4 °C, dried and resuspended in 100 mM Tris buffer pH 8 containing 100 mM NaCl and 1 mM EDTA for further purification. Covalent chromatography was performed on Thiol-sepharose 4B (GE Healthcare, Uppsala, Sweden). The matrix was equilibrated in 100 mM Tris buffer pH 8.0 containing 100 mM NaCl and 1 mM EDTA (20 column volumes (CV)) first, and then the sample was applied. Unbound proteins were eluted with 20 CV of 100 mM Tris buffer pH 8 containing 100 mM NaCl and 1 mM EDTA, while bound protein was eluted by addition of 10 mM L-cysteine to the starting buffer. The homogeneity of purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The concentration of the purified protein was determined using the Bradford method. Bovine serum albumin (BSA) was used as the standard.¹²

Papain activity measurement

The proteolytic activity of the purified papain was tested using BAPNA (*N*^α-benzoyl-DL-arginine 4-nitroanilide hydrochloride, Sigma–Aldrich, Steinheim, Germany) as a substrate in a 96-well microplate (Sarstedt, Numbrecht, Germany) as described in Raskovic *et al.*¹³ Briefly, substrate solution, 100 μ L of 1mM BAPNA in citrate buffer (50 mM pH 6 with 2 mM EDTA and 10 mM L-cysteine) was mixed with an aqueous papain solution (25 μ L) and

incubated at 25 °C. After 60 min, the reaction was stopped by the addition of glacial acetic acid and absorbance at 405 nm was measured.

Thermal inactivation curve

Papain solution (0.5 mg mL⁻¹ in 100 mM Tris buffer pH 8 containing 100 mM NaCl, 1 mM EDTA and 10 mM L-cysteine) was incubated at different temperatures ranging from 15 to 100 °C for 30 min. Samples were allowed to cool to room temperature, and percentage of residual activity was determined in each sample using BAPNA. Measurements were performed in triplicate. The percentage residual activity was calculated relative to the sample that was incubated at 40 °C (maximal activity).

Determination of inactivation rate

Papain solutions were incubated at 60 or 80 °C and aliquots removed at specific intervals (1 min up to 2 h). After cooling to room temperature, the residual activity was measured using the BAPNA assay. The measurements were performed in triplicate.

Determination of the inactivation constants

Inactivation rate constants for papain were determined at four different temperatures selected from the declining part of the inactivation curve (75, 80, 85 and 90 °C). Papain solutions were incubated at an appropriate temperature, and aliquots were taken at specific intervals, ranging between 1 and 30 min. After cooling to room temperature, the samples were assayed with BAPNA. The residual activity was determined in comparison to the activity of the sample before incubation at the respective temperature. A plot of logarithmic residual activity vs. incubation time (expressed in minutes) was produced in order to calculate the rate constants for the inactivation of papain. Rate constant values were calculated from the slopes of the following linear regression:

$$\log (100A/A_{\max}) = -(k/2.303)t \quad (1)$$

where A represents the measured absorbance at 405 nm after certain period of incubation; A_{\max} represents the maximal absorbance before incubation; k represents inactivation rate constant; and t represents the incubation time.¹⁴

Arrhenius plot. Activation energy of papain inactivation was determined by plotting the inactivation constants on the Arrhenius plot. Activation energy was calculated from the slope of Arrhenius plot ($\ln (k / \text{min}^{-1})$ vs. T^{-1} / K^{-1}) in accordance with the equation:

$$\ln k = -E_a/RT + C \quad (2)$$

where R represents the universal gas constant (8.314 J mol⁻¹ K⁻¹), and T represents absolute temperature in K. Slopes and their standard errors were obtained from the linear regression curves.¹⁵

SDS-PAGE and activity staining

Papain samples for SDS-PAGE and activity staining analysis were prepared by mixing 0.5 mg mL⁻¹ protein solution in 100 mM Tris buffer pH 8 containing 100 mM NaCl, 1 mM EDTA and 10 mM L-cysteine with adequate volume of reducing sample buffer (60 mM Tris buffer pH 6.8 containing 25 % glycerol, 1 % SDS, 14.4 mM 2-mercaptoethanol, and 0.1 % bromophenol blue) followed by 30 min incubation at various temperatures (60, 65, 70, 75, 80, 85 and 90 °C). The amount of protein loaded on the gel was 15 µg and 0.1 µg in the case of SDS-PAGE and activity staining, respectively. In the case of SDS-PAGE analysis, the samples were resolved in a discontinuous buffer system with a 4 % stacking gel and 10 % resolving gel in a Hoefer Dual Gel Mighty Small SE 245 vertical electrophoresis system

(Hoefer, Holliston, MA, USA) according to Laemmli.¹⁶ In order to detect in-gel proteolytic activity of papain after SDS–PAGE, zymograms were run according to Felicioli *et al.* with some modifications.¹⁷ Briefly, protein samples were applied onto a 10 % resolving gel co-polymerized with 0.1 % gelatin. After electrophoresis, the gel was incubated in a 100 mM Tris, pH 8 with 100 mM NaCl and 1 mM EDTA for 16 h, followed by staining with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany).

Fourier transform infrared spectroscopy (FT-IR)

Infrared spectra were recorded for various papain samples (incubated for a period of 30 minutes at 25, 60 or 90 °C, with or without the addition of 1 % SDS, the same SDS concentration as in the reducing sample buffer for SDS–PAGE), utilizing Fourier transform infrared spectroscopy with attenuated total reflectance (ATR) at 1 cm⁻¹ resolution (Nicolet 6700 FT-IR, software OMNIC, version 7.0, Thermo Scientific, USA). Papain samples (15 µg) were applied onto a Smart accessory with a diamond crystal (Smart Orbit, Thermo Scientific, USA). The solvent was evaporated under a nitrogen stream in order to obtain a thin ATR film. For each spectrum, 64 scans were collected. The spectrum of the buffer was subtracted from the spectrum of the protein since even water vapor can interfere with the protein absorbance. Criterion for the correctness of subtraction was a flat baseline between 1800 and 2000 cm⁻¹.¹⁸

Spectral analysis. Resolution enhanced spectra were generated by Fourier self-deconvolution function using 13 cm⁻¹ for the full bandwidth at half height (FWHH) and 2.4 for the resolution enhancement factor as described in the study of Byler and Susi.¹⁹ Second-derivative spectra were generated by a seven-point Savitsky–Golay derivative. The positions of the secondary structure peaks were identified from the second-derivative spectra and assigned to specific secondary structures, as described previously.^{19,20} Aggregation specific bands were identified from low frequency bands.^{21–23}

To compare the secondary structure transition in the papain samples incubated for 30 min at 25, 60 or 90 °C, with or without the addition of 1 % SDS, the ratio between the band intensities from the corresponding frequencies of a specific secondary structure and the amide II band maximum, identified at 1520 cm⁻¹, was calculated as described before.^{24,25}

RESULTS AND DISCUSSION

Cooperative thermal inactivation of papain

Fruit papain was purified 8-fold from a commercial preparation in order to remove any inactive protein and colored low molecular weight compounds, which could interfere with the activity estimation assay (Fig. 1). In order to examine the dependence of papain activity and temperature, the residual activity of papain was observed at different temperatures using BAPNA as a substrate. Temperature range was 15–100 °C. After 30 min of incubation at a given temperature, the absorbance of the product was read and an inactivation curve was constructed (Fig. 2). The percentage residual activity was expressed in relation to the activity at 40 °C (maximal activity). The T_{m50} value was found to be 79±2 °C, which is comparable to the T_{m50} value of around 80 °C determined by differential scanning calorimetry.^{11,26} In the incubation temperature region from 60 to 90 °C, the residual activity was reduced from 95 to only 1 %. In this part of the inactivation curve, cooperative loss of activity could be clearly observed due to

denaturation of the protein. Since papain domains unfold almost independently,²⁶ it seems that loss of activity could be attributed to the transition of one domain. Active site residues (Cys-25, His-159 and Asn-178) are located in the cleft formed between these two domains, thus the destabilization of one domain could lead to inactivation.¹¹

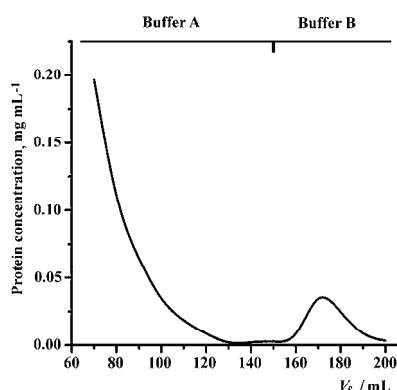


Fig. 1. Chromatographic separation of papain on a thiol-sepharose column. The column was equilibrated with 100 mM Tris buffer pH 8.0 containing 100 mM NaCl and 1 mM EDTA (Buffer A) followed by elution of unbound proteins with the same buffer and elution of papain with the same buffer containing 10 mM cysteine (Buffer B). The chromatography was run at a flow rate of 0.8 mL min⁻¹.

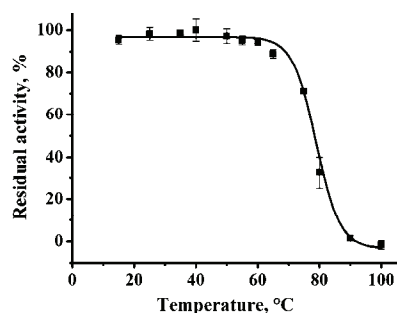


Fig. 2. Thermal inactivation curve of papain determined using BAPNA as a substrate. For creating the curve, A_{405} was monitored as a function of temperature over the appropriate temperature range (15–100 °C). The residual activity was calculated as the percentage of the maximal activity measured at 40 °C.

Papain inactivation rate at 60 and 80 °C

The term kinetic stability is used to describe proteins that are trapped in a specific conformation because of an unusually high unfolding barrier that results in very slow unfolding rates.¹¹ Since inactivation of papain coincided with transition of its tertiary structure, the overall kinetic stability of papain was estimated from its inactivation rates at different temperatures. Papain was incubated at 60 and 80 °C and at specific intervals starting from 1 min up to 2 h, aliquots were removed and the proteolytic activity against BAPNA was assayed. At 80 °C, the activity decline followed first order exponential kinetics (Fig. 3B). When inactivation curve of papain at 60 °C was determined, no decline of activity was registered, indicating stability of the enzyme at 60 °C (Fig. 3A) in the observed time range.

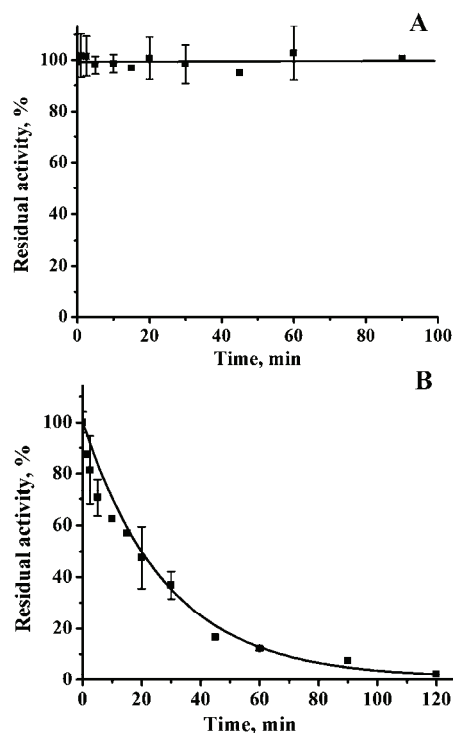


Fig. 3. Kinetics of papain inactivation at: A) 60 and B) 80 °C.

Thermal inactivation of papain

Thermal inactivation of papain was kinetically investigated in the temperature range 75–90 °C. The results are shown in Fig. 4.

First-order exponential curves for dependence of residual activity of papain on incubation time at temperatures in the range 75–90 °C were linearized by plotting the logarithm of the residual activity as a function of incubation time (Fig. 4A). The inactivation rate constants (k / min^{-1}) were calculated from the linear regression analysis and are given in Table I.

The inactivation rate constants increased approximately 20-fold in the temperature range 75–90 °C. Effect of temperature on inactivation rate is illustrated by Arrhenius plot shown in Fig. 4B.

Temperature dependence of the thermal inactivation of papain, expressed by the activation energy ($E_a / \text{kJ mol}^{-1}$) value, is given in Table I. Fruit papain exhibited slightly higher thermal inactivation resistance when compared to stem papain (the obtained E_a value for fruit papain was $223 \pm 16 \text{ kJ mol}^{-1}$, while for stem papain, it was $214 \pm 42 \text{ kJ mol}^{-1}$).²⁷ Recently, it was shown that latex and fruit papain differ in both stability and their catalytic properties under acidic conditions and in the presence of ethanol.³ There is a possibility that fruit and stem papains are not the same enzyme but are isoenzymes. In the case of some other

laticiferous plants (e.g., *Ananas comosus*), different enzymatic profiles could be detected in the stem latex and the fruit regarding molecular weight, amino acid composition, substrate specificity, etc. Thus, stem and fruit bromelains, which belong to the papain family of proteases, could be considered as isoenzymes.²⁸

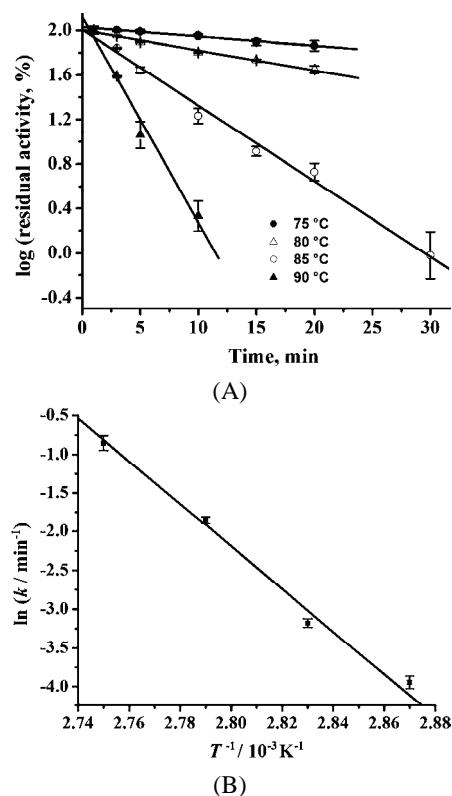


Fig. 4. A) Thermal inactivation of papain in the range between 75 to 90 °C. B) Arrhenius plot showing the temperature dependence of the rate constant of papain inactivation.

TABLE I. Inactivation rate constants (k / min⁻¹) of papain incubated at different temperatures

Temperature, °C	75	80	85	90	E_a / kJ mol ⁻¹
k / min ⁻¹	0.019±0.002	0.042±0.002	0.157±0.007	0.43±0.04	223±16

The E_a value of papain (223±16 kJ mol⁻¹) was considerably higher when compared to enzymes that are less stable in regards to temperature and presence of SDS. The E_a values for several enzymes having overall temperature stability below 50 °C and notorious instability in the presence of SDS were found to be 121,²⁹ 88³⁰ and 167 kJ mol⁻¹,³¹ for carbonic anhydrase, urease and amylase, respectively. In the case of proteolytic enzymes, the inactivation E_a value is very similar to the E_a value of fruit papain. For example, ficin has an inactivation E_a of 210±22 kJ mol⁻¹.²⁷

Papain resistance to SDS

Activity staining (Fig. 5) showed that papain was stable at elevated temperatures (up to 60 °C) in the presence of SDS. At the temperatures higher than 60 °C, a gradual loss of activity was observed. Shift in SDS-PAGE mobility of the native (and active) papain in comparison to the denatured one could be explained by the resistance of papain to SDS denaturation. Since native papain showed resistance to SDS, its net negative charge during electrophoresis was decreased in comparison to the net negative charge of denatured papain (mainly originating from the sulfate group of the detergent).¹⁰ It is noteworthy that papain expressed similar thermal stability regardless of the presence of SDS (Figs. 2 and 5). Since such extraordinary SDS stability of proteins was discussed in terms of the kinetic stability of the β -sheet towards SDS unfolding,¹⁰ the propensities of changes of the certain secondary structure elements in the papain molecule upon incubation at elevated temperatures with and without added detergent were explored in the present study.

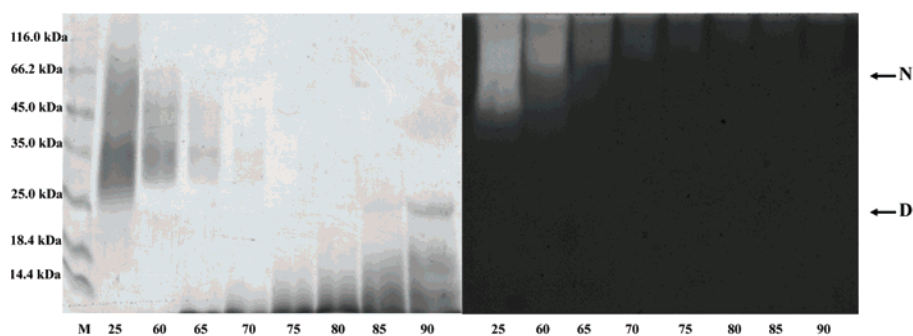


Fig. 5. SDS-PAGE analysis and activity staining towards gelatin of papain incubated at 25 °C and at temperatures from 60 to 90 °C in the presence of 1 % SDS. N – native papain; D – denatured papain.

Changes in secondary structures of papain

FT-IR spectroscopy was employed for monitoring the changes in the secondary structures. The IR spectra of papain incubated at elevated temperatures with or without SDS are shown in Figs. 6 and 7.

FT-IR spectroscopy is well-established method for the analysis of the secondary structures of proteins. The mostly used spectral region for secondary structure analysis is the amide I band (frequency limits: 1600–1700 cm^{-1}), which is almost entirely due to the C=O stretching vibrations of the peptide bonds.³² Specific maxima within amide I region were identified from second-derivative spectra and assigned to secondary structures: α -helix 1654 cm^{-1} and β -sheet 1632 cm^{-1} according to Byler and Susi.¹⁹ Goormaghtigh *et al.*²⁰ reported that 1641

cm^{-1} is a random coil specific frequency for papain, while Byler and Susi,¹⁹ found it at 1646 cm^{-1} . In the present study, the random coil band was identified at 1643 cm^{-1} . An aggregation specific intermolecular β -sheet conformation was identified at a low frequency at around 1612 cm^{-1} .^{21–23}

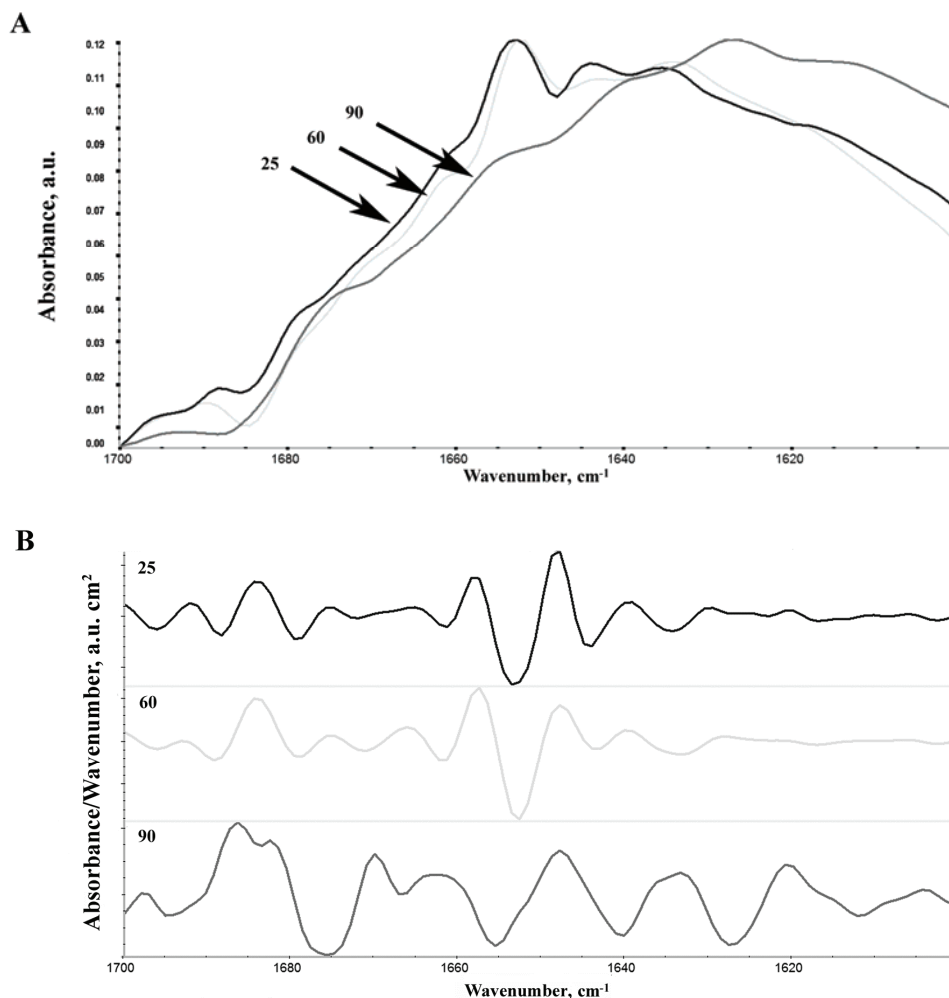


Fig. 6. A) Fourier self-deconvolution IR spectra and B) second derivative spectra of papain incubated at 25, 60 and 90 °C.

Changes in band intensity ratios of certain secondary structures identified within amide I region and amide II band in the IR spectra of papain incubated at elevated temperatures with or without added SDS are shown in Fig. 8.

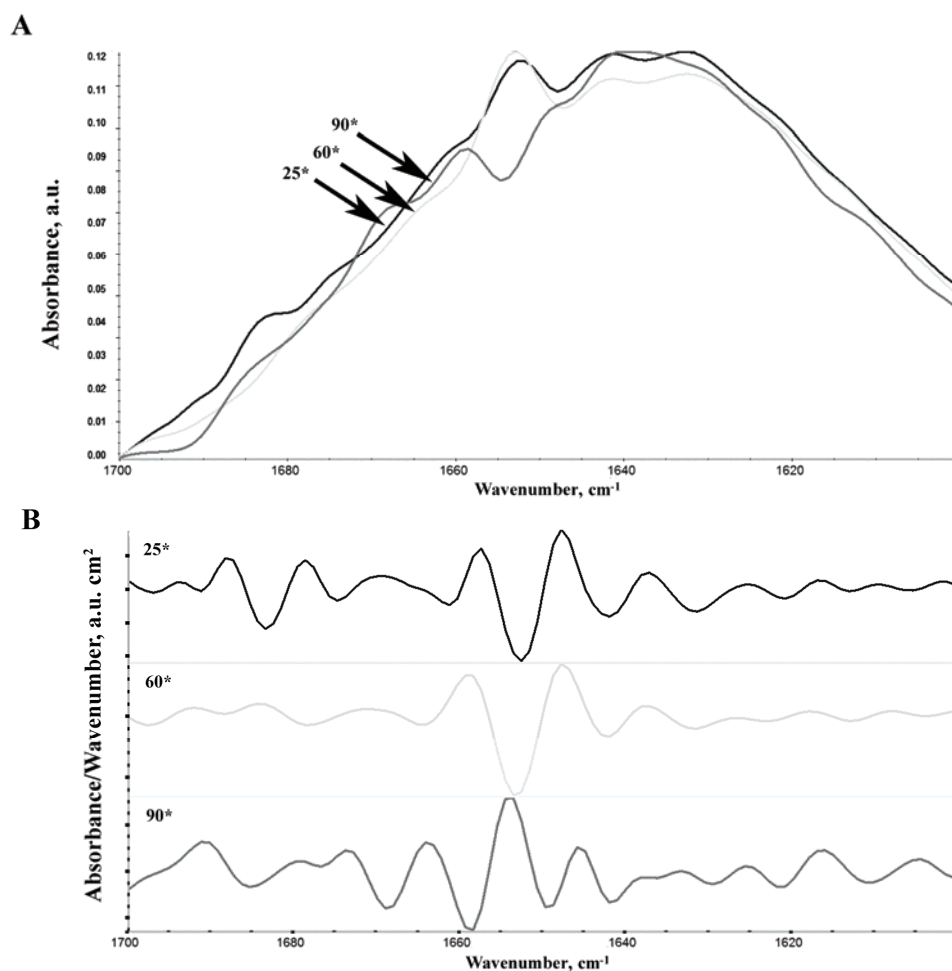


Fig. 7. A) Fourier self-deconvolution IR spectra and B) second derivative spectra of papain incubated at 25, 60 and 90 °C with addition of SDS (25*, 60* and 90*).

Denaturation of papain induced by heat (90 °C) led to lowered contents of native-like structures (ordered: α -helix; and unordered – random coil), while it accelerated the formation of an aggregation specific intermolecular β -sheet (Fig. 8). Several authors reported that frequencies 1614–1622 cm⁻¹ and sometimes above 1680 cm⁻¹ are aggregation specific frequencies in all α -helix rich,^{22,33} β -sheet rich proteins,²¹ and mixed fold proteins,²³ and that they appear due to the formation of intermolecular antiparallel β -sheets prior to aggregation. It was shown in the case of several proteins, including myoglobin,²² transthyretin,³⁴ β -lactoglobulin,²¹ human growth hormone, human interferon- α -2b²² and chymotrypsinogen A²³ that heat denaturation induced changes in the amide I region,

corresponding to shift of the intensities of frequency bands of native-like structures toward aggregation specific β -sheet frequency bands. Furthermore, the formation of the aggregates was lowered in the presence of SDS at elevated temperatures (60 and 90 °C, Fig. 8). However, in the case of added detergent it is apparent that elevated temperatures (especially at 90 °C) accelerated the formation of unordered structures, while lowering the content of α -helix structures. Changes in the β -sheets induced by the presence of SDS were not so pronounced, indicating that denaturation of papain by a combination of heat and detergent started within the α -helical domain. Lowered stability of the α -helical domain of papain in comparison to the β -sheet domain was recently reported in case of acid denaturation of papain.³⁵

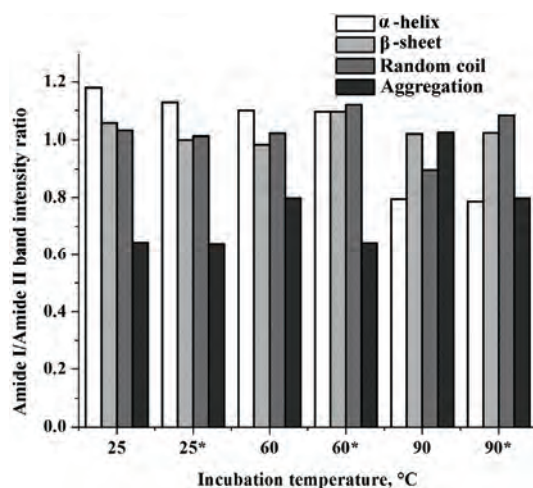


Fig. 8. Changes in intensity ratios of secondary structure specific bands in the IR spectra of papain incubated at 25, 60 and 90 °C (25, 60 and 90) and with addition of SDS (25*, 60* and 90*). The amide I/ amide II band intensity ratios were calculated using the intensity of secondary structure specific band in the amide I region (1654 cm^{-1} α -helix; 1632 cm^{-1} β -sheet; 1643 cm^{-1} random coil; 1612 cm^{-1} aggregation specific intermolecular β -sheet) and the amide II maximum intensity at 1520 cm^{-1} .

The explanation of SDS resistance of kinetically stable proteins is based on two assumptions. SDS binding appears to rely on transitions between protein conformations, moments of weakness in which the protein is susceptible to SDS binding, thus leading to entrapment.¹⁰ On the other hand, kinetically stable proteins are characterized by unusually low structural flexibility, which lead to suppression of partial unfolding.³⁶ Thus, kinetically stable proteins, papain among them, infrequently assume open conformations and therefore are resistant to SDS unless provided with energy in the form of heat. With the exception of the papain family of proteases, most of the kinetically stable and SDS resistant proteins exhibit a tight β -sheet fold and tend to oligomerize.¹⁰ However, despite having mixed α/β fold, papain is not the exception to the rule. The obtained results suggest that the α -helical domain of papain is more prone to unfolding at elevated temperatures and in the presence of SDS than the β -sheet rich domain.

CONCLUSIONS

In this study, for the first time, the differential SDS resistance of the α -helical N-terminal domain of papain and the β -sheet rich C-terminal domain is reported. Denaturation of papain by a combination of heat and SDS led to a reduction of the IR band assigned to α -helix, while preserving the β -sheet content. However, regardless of fruit papain denaturation at 90 °C, the protein exhibited considerable thermal stability and SDS resistance.

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ИЗВОД

ДОМЕН БОГАТ β -ПЛОЧИЦАМА ЈЕ ОДГОВОРАН ЗА КИНЕТИЧКУ СТАБИЛНОСТ
ПАПАИНА ПРИ ДЕНАТУРАЦИЈИ ИЗАЗВАНОЈ ПОВИШЕНОМ ТЕМПЕРАТУРОМ И
НАТРИЈУМ-ДОДЕЦИЛ-СУЛФАТОМ

БРАНКИЦА РАШКОВИЋ, НИКОЛИНА БАБИЋ, ЈЕЛЕНА КОРАЋ И НАТАЛИЈА ПОЛОВИЋ

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Папаин је протеаза која у својој структури поседује два домена од којих је један богат α -хеликсом, а други β -плочицом. Ова два домена се при денатурацији готово независно развијају. За папаин изолован из стабла папаје показана је знатна температурна стабилност, као и отпорност на натријум-додецил-сулфат (SDS). Међутим, подложност сваког од домена да се развијају услед денатурације изазване повишеном температуром или SDS, још увек није проучена. У овом раду је показано да папаин из плода папаје испољава благо повишену отпорност на термалну инактивацију у поређењу са папаином из стабла папаје са прилично високом вредношћу енергије активације (E_a) која износи $223 \pm 16 \text{ kJ mol}^{-1}$ и T_m 50 вредношћу од $79 \pm 2 \text{ }^\circ\text{C}$. SDS-PAGE анализом и зимограмом одређена је отпорност папаина плода папаје на SDS. Изгледа да протеин задржава активност у присуству SDS-а све док се не обезбеди довољна количина енергије у виду топлоте која ће довести до развијања протеина. Употребом Фурије трансформисане инфрацрвене спектроскопије (FT-IR) показано је да је домен папаина плода папаје богат α -хеликсом подложнији развијању на повишеним температурама и у присуству SDS од домена богатог β -плочицама. Термална денатурација протеина без присуства детергента доводи до бржег формирања интермолекуларске β -плочице специфичне за агрегацију протеина када се упореди са нативним протеином. Приказани резултати поседују фундаментални значај, али су веома важни и у контексту комерцијалне примене папаина.

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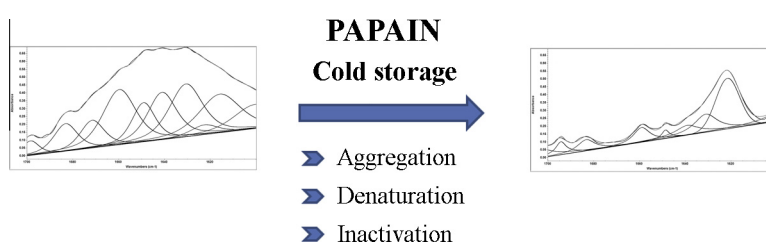
Fourier transform infrared spectroscopy provides an evidence of papain denaturation and aggregation during cold storage

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HIGHLIGHTS

- Papain was the most stabile from 45 to 60 °C with ΔG°_{321} 13.9 kJ/mol and T_m value 84 °C.
- Cold storage of papain gave an increase of 40% of intermolecular β -sheet content.
- Six freeze–thaw cycles induced 75% activity loss due to denaturation and aggregation.
- Autoproteolysis of papain did not cause significant loss of the protein activity.

GRAPHICAL ABSTRACT



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ABSTRACT

Papain is a cysteine protease with wide substrate specificity and many applications. Despite its widespread applications, cold stability of papain has never been studied. Here, we used differential spectroscopy to monitor thermal denaturation process. Papain was the most stabile from 45 °C to 60 °C with ΔG°_{321} of 13.9 ± 0.3 kJ/mol and T_m value of 84 ± 1 °C. After cold storage, papain lost parts of its native secondary structures elements which gave an increase of 40% of intermolecular β -sheet content (band maximum detected at frequency of 1621 cm^{-1} in Fourier transform infrared (FT-IR) spectrum) indicating the presence of secondary structures necessary for aggregation. The presence of protein aggregates after cold storage was also proven by analytical size exclusion chromatography. After six freeze–thaw cycles around 75% of starting enzyme activity of papain was lost due to cold denaturation and aggregation of unfolded protein. Autoproteolysis of papain did not cause significant loss of the protein activity. Upon the cold storage, papain underwent structural rearrangements and aggregation that correspond to other cold denatured proteins, rather than autoproteolysis which could have the commercial importance for the growing polypeptide based industry.

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Introduction

Papain is a cysteine protease (EC 3.4.22.2) with wide substrate specificity and many applications. It has been isolated from the latex of the papaya fruit. The preprotein consists of 345 amino

acids and it is secreted as zymogene [1]. After the cleavage of the activation peptide, the mature enzyme contains 212 amino acid residues organized in two domains. The N-terminal domain has mainly α -helical structure while C-terminal domain has antiparallel β -sheet fold [2].

Papain is extensively used as meat tenderizer [3], in dental caries removal procedures [4], for preparation of clinically relevant antibody fragments [5], as a cell dissociation/debris removal agent

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[6], as a component in cosmetics [7] and detergents [8]. The main limitation in usage of proteolytic enzymes in many applications is their temperature stability, both at elevated temperatures and during freezing. It has been already shown that papain is stable at elevated temperatures with T_m value at $83 \pm 1^\circ\text{C}$ [9]. However, cold stability (especially after freezing and thawing) of papain has never been studied.

During freezing and storage of proteins in frozen state, several factors can impact protein stability. Cold denaturation of globular proteins has been detected in several cases especially at high pressure [10–13]. Theoretically, cold denaturation should happen as a consequence of changes in interactions between water and protein molecules at low temperatures thus should represent a universal phenomenon. However, cold denaturation is difficult to study since most proteins have denaturation points below 0°C [14]. Further, the denaturation of a protein during freezing could also occur as a consequence of changes in solute concentration due to ice formation and changes of pH value in the protein surrounding [15]. Freeze–thaw cycles are routinely used as a tool to determine the combined effects of these different freezing related stresses on protein stability [15,16].

The aim of this paper was to study cold storage stability of papain. We describe a significant loss of papain activity as a consequence of cold storage. This phenomenon could be of importance for industrial conservation of the enzyme since cold storage of papain leads to its denaturation and aggregation.

Materials and methods

Papain purification

In order to remove inactive/denatured protein and low molecular weight compounds, papain was purified from commercial papain preparation (The British Drug House Ltd, London, England). Papain was extracted from dry powder with 100 mM Tris buffer pH 8 containing 100 mM NaCl and 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA) for 30 min at 10°C . Extract was centrifuged for 15 min at 4000 g at 10°C and obtained supernatant was further used. Papain was precipitated from supernatant by addition of 2 volumes of ice cold acetone followed by incubation at -20°C for 30 min. The precipitate was separated by centrifugation at 10,000 g for 5 min at 4°C , dried and resuspended in 100 mM Tris buffer pH 8 containing 100 mM NaCl and 1 mM EDTA for further purification. Covalent chromatography was performed on Thiol-Sepharose 4B (GE Healthcare, Uppsala, Sweden). Matrix was equilibrated in 100 mM Tris buffer pH 8.0 containing 100 mM NaCl and 1 mM EDTA (20 column volumes (CV)) first, then the sample was applied. Unbound proteins were eluted with 20 CV of 100 mM Tris buffer pH 8 containing 100 mM NaCl and 1 mM EDTA, while bound protein was eluted by addition of 10 mM L-cysteine to the starting buffer. The homogeneity of purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The activity was analyzed by zymography and N α -Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) hydrolysis. Concentration of the purified protein was determined using Bradford method. Bovine serum albumin (BSA) was used as standard [17].

SDS PAGE and zymogram

For in gel analysis of proteolytic activity detection papain was resolved under reducing conditions in a discontinuous buffer system SDS–PAGE with a 4% stacking gel and 12% resolving gel in a Hoefer Dual Gel Mighty Small SE 245 vertical electrophoresis

system (Hoefer, Holliston, USA) according to Laemmli [18]. The amount of 2.5 $\mu\text{g}/\text{mm}$ of the protein was applied on the gel. To detect proteolytic activity of papain after SDS–PAGE, zymograms were developed according to Felicioli et al. [19] with some modifications. Briefly, after incubation in non reducing sample buffer (1 h at 60°C and 5 min at 95°C), protein samples were applied onto a 12% resolving gel co-polymerized with 0.1% gelatin for SDS–PAGE analysis. After electrophoresis, the gel was incubated in a 100 mM Tris, pH 8 with 100 mM NaCl 1 and mM EDTA for 16 h, followed by staining with Coomassie Brilliant Blue R-250 (CBB) (Serva, Heidelberg, Germany).

Trypsin mass fingerprinting

Protein spot was excised from CBB stained gel to perform in-gel trypsin digestion according to the manufacturer instructions. Obtained peptides were injected onto a reversed phase C18 column (ACQUITY UPLC BEH 130, 1.7 μm , 2.1×50 mm, Waters, Milford, MA, USA) installed on Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) and separated using acetonitrile gradient (0–50% for 40 min with 0.1% formic acid). After detection with photodiode array (UV) at 280 nm mass spectra were recorded on triple-quadrupole mass spectrometer (ACQUITY TQD, Waters, Milford, MA, USA) in positive ion mode with capillary voltage of 3 kV and drying gas flow rate of 8.3 L/min at 250°C . The scan range was set from 400 to 2000 m/z. The peptide masses were searched against the SwissProt protein sequence database using the MASCOT program.

Differential UV spectroscopy

UV absorbance measurements were performed on Evolution 300 spectrophotometer (Thermo Fisher Scientific, Madison, USA) using matched 1 cm path length quartz cuvettes. The spectrophotometer was equipped with an electrothermal temperature controller which provides thermal programmability for the multiple cell units so that the absorbance measurements can be performed directly as a function of temperature. Spectra of native (room temperature) or thermally denatured papain (99°C) were recorded from 230 nm to 300 nm in 50 mM Tris buffer pH 8.0. Papain concentration was 1 mg/mL. Spectra of buffer only served as a blank. Equilibrium thermal unfolding of papain was monitored by recording absorbance at 230, 267 and 286 nm as a function of temperature over the appropriate temperature range (5 – 99°C). The UV difference spectrum was calculated by subtraction of absorbances of fully native and fully denatured papain at all wavelengths in the recorded region as described by Hatley and Franks [20]. The wavelength 267 nm was chosen for further analysis as the maximal difference between the denatured and the native protein spectra was observed at this wavelength. The heating rate was $0.5^\circ\text{C min}^{-1}$.

Using UV spectroscopy we were able to calculate Gibbs free energy (ΔG°) of papain denaturation at different temperatures. Fraction of thermally denatured papain (F_d) was calculated using equation:

$$F_d = (Y_N - Y_{\text{obs}}) \times (Y_N - Y_D)^{-1} \quad (1)$$

In the given equation Y_{obs} represents the observed value of absorbance, while Y_N and Y_D represents absorbance values characteristic of fully native and fully denatured papain, respectively.

From the following equation, equilibrium constant of the process (K) could be obtained:

$$K = F_d(1 - F_d)^{-1} \quad (2)$$

Calculated values for F_d and K were further used for Gibbs free energy calculation according to the following equation:

$$\Delta G^\circ = -RT \ln K \quad (3)$$

where R is universal gas constant and T is the absolute temperature [21].

Differential scanning calorimetry (DSC)

All thermograms were acquired on a DSC Q1000 series (TA Instruments, New Castle, Delaware) with an auto sampler and a refrigerated cooling system (RCS, TA Instruments). Approximately 7 mg of the protein solution (10 mg/ml) in 50 mM Tris buffer pH 8.0 was weighed into an aluminum pan and hermetically sealed with an aluminum cover. Reference pan was filled with adequate weight of buffer solution. Sample was equilibrated on 10 °C and then heated to 90 °C at a heating rate of 1 °C/min. Temperature maximum (T_m) of the endothermal transition corresponding to protein denaturation was determined by TA Instruments Universal Analysis 2000 software (version 4.1D).

Papain activity measurement

Biological activity of purified papain was tested using BAPNA (Sigma–Aldrich, Steinheim, Germany) as a substrate in a 96-well microtitre plate (Sarstedt, Numbrecht, Germany) as described in Raskovic et al. [22] with some modifications. Briefly, 100 μ L substrate solution (1 mM BAPNA in 100 mM Tris buffer pH 8.0 with 1 mM EDTA and 10 mM L-cysteine) was mixed with aqueous papain solution (25 μ L) incubated at 25 °C. After 60 min the reaction was stopped by the addition of glacial acetic acid and the absorbance at 405 nm was measured.

Freeze–thaw cycles

One hundred microliters of purified papain was frozen at –20 °C in 1.5 mL centrifuge tubes. After one hour the samples were thawed at the room temperature. Freeze–thaw cycles were repeated up to six times. Relative activity of papain was determined after each cycle as activity remaining in the sample. Starting activity detected in the sample was considered as 100%. All experiments were repeated three times.

Autoproteolysis measurement

Autoproteolysis was measured as described previously [23] with some modifications. To test the autoproteolysis 10 μ L of 0.2 mg/mL papain solution was used. An aliquot was immediately tested for autoproteolysis. Solutions were subjected to 6 freeze–thaw cycles as already described. After each cycle, an aliquot (10 μ L) was mixed with 200 μ L of CBB (0.125% Coomassie Brilliant Blue G-250 in 24% ethanol and 48% phosphoric acid). After 5 min, the absorbance at 620 nm was measured. The assay was performed in triplicate.

Reverse-phase chromatography analysis of autoproteolytic products

Reverse-phase chromatography of the untreated papain and freeze–thawed papain samples at concentration 1 mg/mL was performed using an Äkta Purifier 10 System (GE Healthcare, Uppsala, Sweden) on a Discovery® BIO Wide Pore C5-5 10 cm \times 4.6 mm, 5 μ m (Supelco, Bellefonte, PA, USA) column. The protein was eluted using an acetonitrile gradient (0–90% for 10 column volumes with 0.1% trifluoroacetic acid).

Secondary structures determination – Fourier transform infrared spectroscopy

Infrared spectra were recorded for all: native, thermally treated (5 min at 95 °C) and cold stored papain (freeze–thawed for 6 times) using Fourier transform infrared spectroscopy (FT-IR) with an attenuated total reflectance (ATR) at 1 cm^{-1} resolution (Nicolet 6700 FT-IR, software OMNIC, Version 7.0, Thermo Scientific, USA). Concentration of the solution of papain samples was 10 mg/mL. Ten microliters of these solutions were applied onto a Smart accessory with diamond crystal (Smart Orbit, Thermo Scientific, USA). Solvent was evaporated by the nitrogen stream in order to obtain thin ATR film. For each spectrum, 64 scans were collected. The spectrum of the buffer was subtracted from the spectrum of protein since it is known to interfere with the protein absorbance in the amide I region. After subtraction, spectrum was smoothed in order to remove the noise (factor of 6.75 cm^{-1}). Criteria for the correctness of subtraction were removal of the band near 2200 cm^{-1} . The region between 1700 cm^{-1} and 1600 cm^{-1} (the amide I region) was fitted. The procedure of curve-fitting was used to decompose the original amide I spectra to its Gaussian–Lorentzian curve constituents that would be assigned to certain structural features. The contribution of each curve to the amide I band was assessed by integrating the area under the curve, and then normalizing it to the total area under the amide I band.

Assignment of secondary structures for all the observed peaks was done according to previously published guidelines [24–27].

Monitoring of aggregation and estimation of molecular weight of papain by size exclusion chromatography

Analytical size exclusion chromatography was performed using Äkta Purifier 10 System (GE Healthcare, Uppsala, Sweden) on Superdex 75 PC 3.2/30 column. Column was equilibrated with 100 mM Tris buffer pH 8 containing 100 mM NaCl and 1 mM EDTA. Papain solution (10 μ L, concentration 1 mg/mL) was applied to the column at flow rate 0.05 μ L/min. The calibration of the column was performed using the mixture of gel filtration molecular weight standards (1 mg/mL each): bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), cytochrome c (14 kDa) and aprotinin (6.5 kDa) (Sigma–Aldrich, Steinheim, Germany). To determine void volume, blue dextran (Sigma–Aldrich, Steinheim, Germany) was used.

Elution volume of molecular weight standards was used to create calibration chart ($V_e/V_t = f(\log Mw)$). Molecular weight of papain was estimated from the calibration chart.

Results

Papain purification

Due to interference of low molecular weight contaminants in spectroscopic methods [28], we first sought to purify papain. Acetone precipitation and covalent chromatography were used for the purification of papain from commercial preparation. Protein concentration was determined using Bradford method, while activity was monitored with BAPNA as a substrate. Acetone precipitation did not cause loss of activity since specific activity remained the same (1.12 U/mg) as in crude papain extract (Table 1).

However, pigments and low molecular weight contaminants were removed rendering the papain solution colorless. Covalent chromatography enabled further purification of the protein, 7.9 times when compared to the starting sample (Table 1, Fig. 1A).

Table 1
Purification of papain.

	Activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	Purification degree
Crude extract	83.3 ± 0.5	15.3 ± 0.2	1.13	1
Acetone precipitation	73 ± 1	13.6 ± 0.5	1.12	0.99
Covalent chromatography	3.9 ± 0.1	0.091 ± 0.002	9.0	7.9

Purification and activity was also monitored by SDS–PAGE and zymogram (Fig. 1B). Samples incubated at 60 °C retained activity while it was lost in the samples incubated at 95 °C. Slower migration of papain was visible in all the bands incubated at 60 °C in comparison to those incubated at 95 °C.

Papain identification by TMF

After SDS–PAGE and CBB staining of protein isolated by acetone precipitation and covalent chromatography, we identified the isolated protein by proteomic analysis after trypsin digestion. According to Mascot, protein scores greater than 70 were considered as significant ($p < 0.05$). Papain was identified by 12 matching peptides, with sequence coverage of 27%, as the only significant hit (Mascot score was 116) (Fig. 1C).

Thermal denaturation of papain

In UV absorption spectra from 255 nm to 300 nm (Fig. 2A) a clear difference between native and denatured molecule of papain was observed. Absorption extinction coefficient (ϵ) maximum of native molecule was at 279 nm ($58.332 \text{ cm}^{-1} \text{ mM}^{-1}$), while it was shifted toward shorter wavelengths for the denatured molecule (275 nm, $63.855 \text{ cm}^{-1} \text{ mM}^{-1}$).

For observing differences in UV spectra between native and denatured molecule a differential UV spectra was calculated (Fig. 2B) as described by Hatley and Franks [20]. The greatest difference in the extinction coefficient was observed at 267 nm and it was $\Delta\epsilon = -8.912 \text{ cm}^{-1} \text{ mM}^{-1}$. This wavelength was used for monitoring papain denaturation in further experiments.

In order to examine thermal denaturation curve of papain, absorbance at 267 nm was observed in temperature range from 5–99 °C. Papain was incubated at given temperature for 2 min before the measurement was performed. Percentage of native structure was expressed in relation to A_{267} at 57 °C (Fig. 2C). Papain is the most stable from 45 °C to 60 °C, with the maximum at 57 °C, as observed from the denaturation curve. From 60 °C to 95 °C percent of native structure is reduced from 99.5% to only 0.5%. In a part of the curve below 45 °C reduction in percent of native structure can be noted as well, and at 5 °C, 15% reduction was observed.

Thermal denaturation T_m value is determined to be 84 ± 1 °C. The same value was determined by differential scanning calorimetry (Supplementary Fig. 1).

Thermodynamics parameter (ΔG°) of papain denaturation process was calculated for 5 °C, 25 °C, 48 °C and 60 °C as described in Yousefi et al. [21]. Results are presented in Table 2.

Cold storage stability of papain

Activity of papain after cold storage at -20 °C was observed using BAPNA as a substrate (Fig. 3). Residual activity was determined after each of 6 freeze and thaw cycles. After 6th cycle about 75% of activity was lost.

Papain autoproteolysis

Papain autoproteolysis during 6 freeze–thaw cycles was monitored using an assay based on CBB binding (Fig. 4A) and to estimate

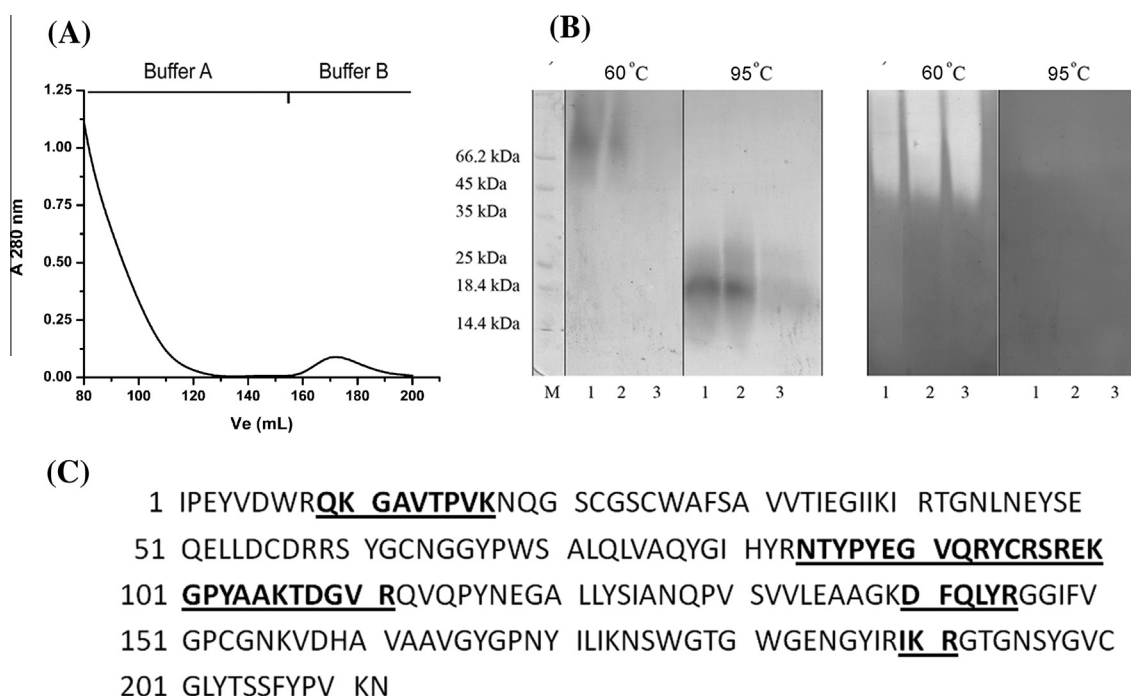


Fig. 1. (A) Chromatographic separation of papain on Thiol-Sepharose column. Column was equilibrated with 100 mM Tris buffer pH 8.0 containing 100 mM NaCl and 1 mM EDTA (Buffer A) followed by elution with the same buffer containing 10 mM cysteine (Buffer B). Chromatography was run with a flow rate of 0.8 mL/min. (B) SDS–PAGE analysis and activity staining toward gelatin of each fraction collected during papain purification: 1 – starting commercial papain extract, 2 – acetone precipitate, 3 – Thiol-Sepharose chromatography bound protein and M – molecular weight markers; (C) peptide mass fingerprint of the isolated papain, identified peptides are bolded and underlined.

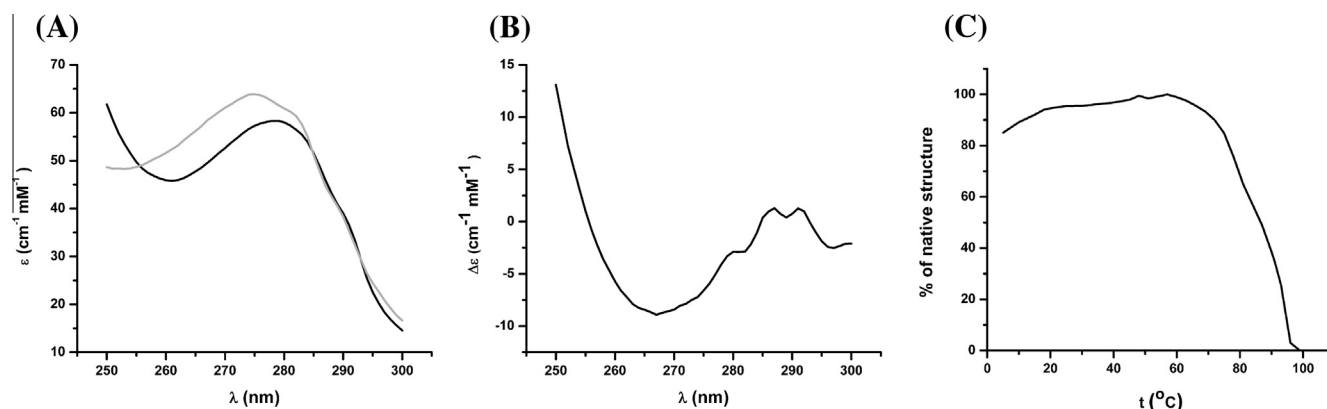


Fig. 2. (A) UV absorption spectra from 255 nm to 300 nm of native papain (black line) and denatured papain (gray line); (B) differential UV absorption spectrum; (C) thermal denaturation curve of papain incubated at lowered and elevated temperatures. For creating the curve A_{267} was monitored as a function of temperature over the appropriate temperature range (5–99 °C). The heating rate was 0.5 °C min⁻¹. The percentage of native structure was calculated as the percentage of maximal change of the extinction coefficient ($\Delta\epsilon$).

Table 2

The Gibbs free energy change (ΔG°) of thermal stability of papain. ΔG° was calculated from the equation $\Delta G^\circ = -RT \ln K$ where R is the universal gas constant and T is the absolute temperature.

	ΔG°_{278} (kJ/mol)	ΔG°_{298} (kJ/mol)	ΔG°_{321} (kJ/mol)	ΔG°_{333} (kJ/mol)
Papain	3.2 ± 0.1	7.5 ± 0.3	13.9 ± 0.3	12.5 ± 0.4

the damage to papain, reverse phase high performance liquid chromatography (RP-HPLC) was performed (Fig. 4B).

We did not detect significant autoproteolysis of papain both in the CBB-binding based assay and in RP-HPLC.

Secondary structure determination

Infrared data were obtained for all: native, thermally treated and cold stored papain. The changes in the Savitzki–Golay second derivative spectra (amide I region) are presented in Fig. 5.

In the second derivative spectrum of native papain several important bands could be observed, especially in the region that could be attributed to most important ordered secondary structures with the maxima at frequencies 1630, 1641, 1648, 1659, 1671, 1673, 1683 and 1697 cm⁻¹ (Fig. 5A). The most prominent change in the second derivative spectrum of thermally treated

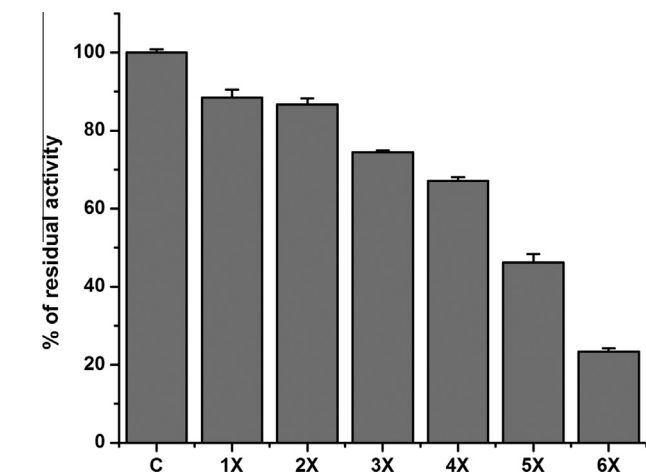


Fig. 3. Activity of papain during six freeze–thaw cycles. Activity of starting papain sample (C) is considered as 100%, and the measured activity after each freeze–thaw cycle (1X–6X) is expressed as a percent of starting activity. Data represent the mean of three separate experiments.

papain (Fig. 5B) is the disappearance of 1648 cm⁻¹ band that could be attributed to α -helix [24]. The second derivative spectrum of cold stored papain differed from native papain spectrum since the most prominent maximum is shifted to 1621 cm⁻¹ indicating the presence of intermolecular β -sheet (Fig. 5C) [26,27].

After decomposition of amide I region of the FT-IR spectra (Fig. 6), peaks were assigned to specific secondary structures (Table 3), and the relative percentage of each secondary structure was calculated using the contribution of integrated intensities of bands (areas) to the total amide I band area. The content of secondary structures in X-ray determined tertiary structure of papain was calculated from UniProt data bank entry (P00784 (PAPA1_CARPA)). The results are presented in Table 4.

The weak 1610–1614 cm⁻¹ band contribution in native state and thermally denatured protein that arises from side-chain vibration [26] was not included in secondary structure estimation. It had also been subtracted from the overlapping band at 1618–1623 cm⁻¹.

Goormaghtigh et al. assigned frequencies 1660–1670 cm⁻¹ in native papain spectrum to turn structures [24]. However, the study of Vedantham et al. [25] showed that bands at frequencies 1665–1676 cm⁻¹ correspond to unordered helix. In this work, assignment of 1672 cm⁻¹ band to unordered helix (Table 3) rather to the turn structure provides better concurrence of estimated secondary structure content of the native papain to the content calculated from X-ray data (Table 4) then the one presented before [24].

Thermal denaturation of papain led to increase in content of β -sheet structures and to decrease of α -helix and unordered structures. Nevertheless, the most pronounced changes in native-like content of secondary structures are induced by papain cold storage (Table 4).

Aggregation of papain during cold storage

Aggregation of cold stored papain was monitored by size exclusion chromatography. The chromatogram of native papain reveals one major peak of molecular weight 24.0 kDa. In the chromatogram of cold stored protein, peak representing native papain was smaller, while the majority of protein present in the sample eluted in the void volume of the column (Fig. 7).

Discussion

To investigate papain stability after cold storage, we first purified commercial papain in order to remove inactive protease and

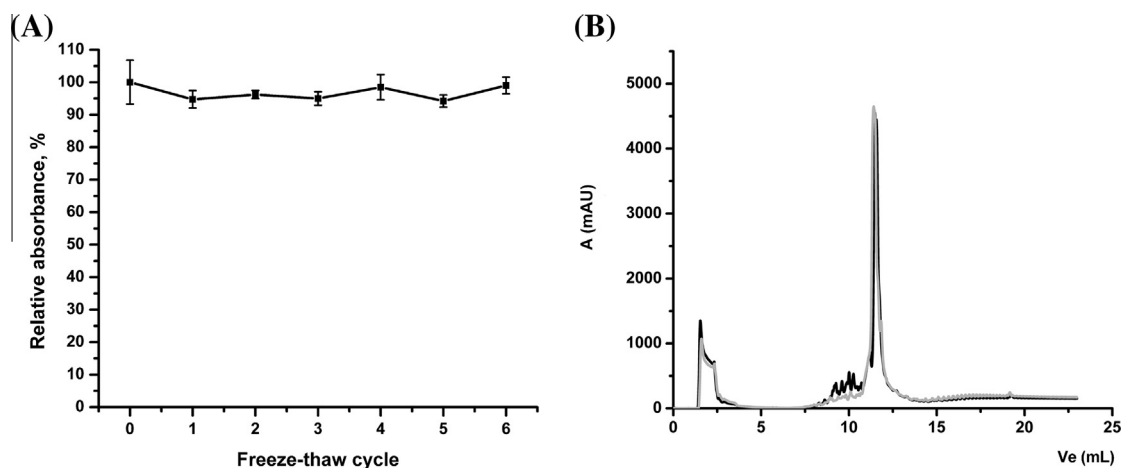


Fig. 4. (A) Autoproteolysis of papain during 6 freeze–thaw cycles. The absorbance of each sample is shown as a percentage of the initial absorbance. (B) RP-HPLC of native (black line) and cold stored papain (gray line).

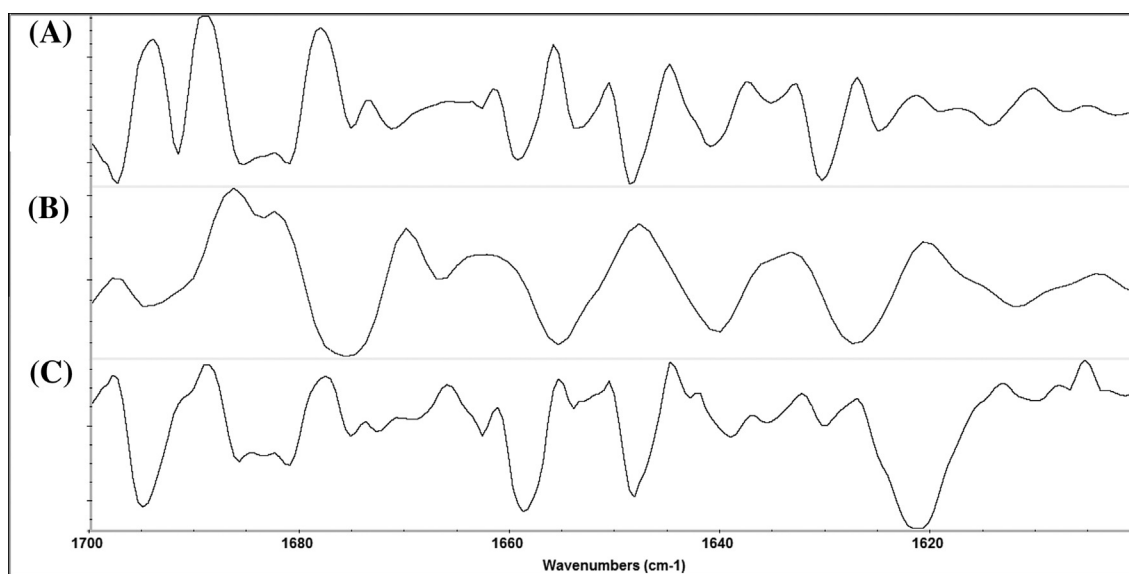


Fig. 5. Second derivative FT-IR spectrum of: (A) native papain; (B) thermally treated papain and (C) cold stored papain.

low molecular weight substances that could interfere with spectroscopic methods. Differential spectroscopy (Fig. 2C, Table 2) and SDS–PAGE zymography (Fig. 1B) showed that papain was stable at elevated temperatures (up to 60 °C). Shift in SDS–PAGE mobility of the native (and active) papain in comparison to denatured one could be explained as a consequence of papain resistance to SDS denaturation. Since, SDS binding to the native molecule of papain was decreased, papain was not completely denatured and its net negative charge was decreased, so shift in SDS–PAGE mobility was very pronounced when compared to completely denatured protein [29]. At 95 °C, high temperature contributes to the denaturation of papain so the protein become completely linearized and fully covered with SDS, which affected its electrophoretic mobility. In addition, molecular weight of the native enzyme determined by size exclusion chromatography confirmed that papain was in a form of monomer, excluding possible oligomerization under native conditions (Fig. 7).

In UV region from 230 nm to 300 nm aromatic amino acids are absorbing and their extinction coefficients are largely dependent upon their mobility and their environments. This is affected by the conformation of the protein, and in native molecule aromatic

amino acid residues are usually placed inside hydrophobic core of the protein. During denaturation their extinction coefficient changes consequently affecting the spectra of the entire protein. Differential UV spectrometry has been extensively used in studying protein denaturation [30–32].

The overall problem in monitoring of cold denaturation of proteins is present due to ice formation at low temperatures which interfere with standard methods used for monitoring protein denaturation [14]. However, we were able to capture the beginning of cold denaturation process using differential spectroscopy. The validity of this approach was proven by T_m value determined for the papain denaturation at elevated temperatures, which is comparable to the previously published one (Fig. 2C). Sathish et al. has recently reported T_m value of 83 ± 1 °C for papain determined by differential scanning calorimetry [9], which is in good agreement with the value that we determined by both methods. In this part of the denaturation curve cooperative denaturation mechanism can be clearly observed. Cold denaturation could also be monitored by measuring A_{230} (data not shown) as recently described [32]. However, we were unable to capture cold transition point using differential scanning calorimetry (Supplementary Fig. 1).

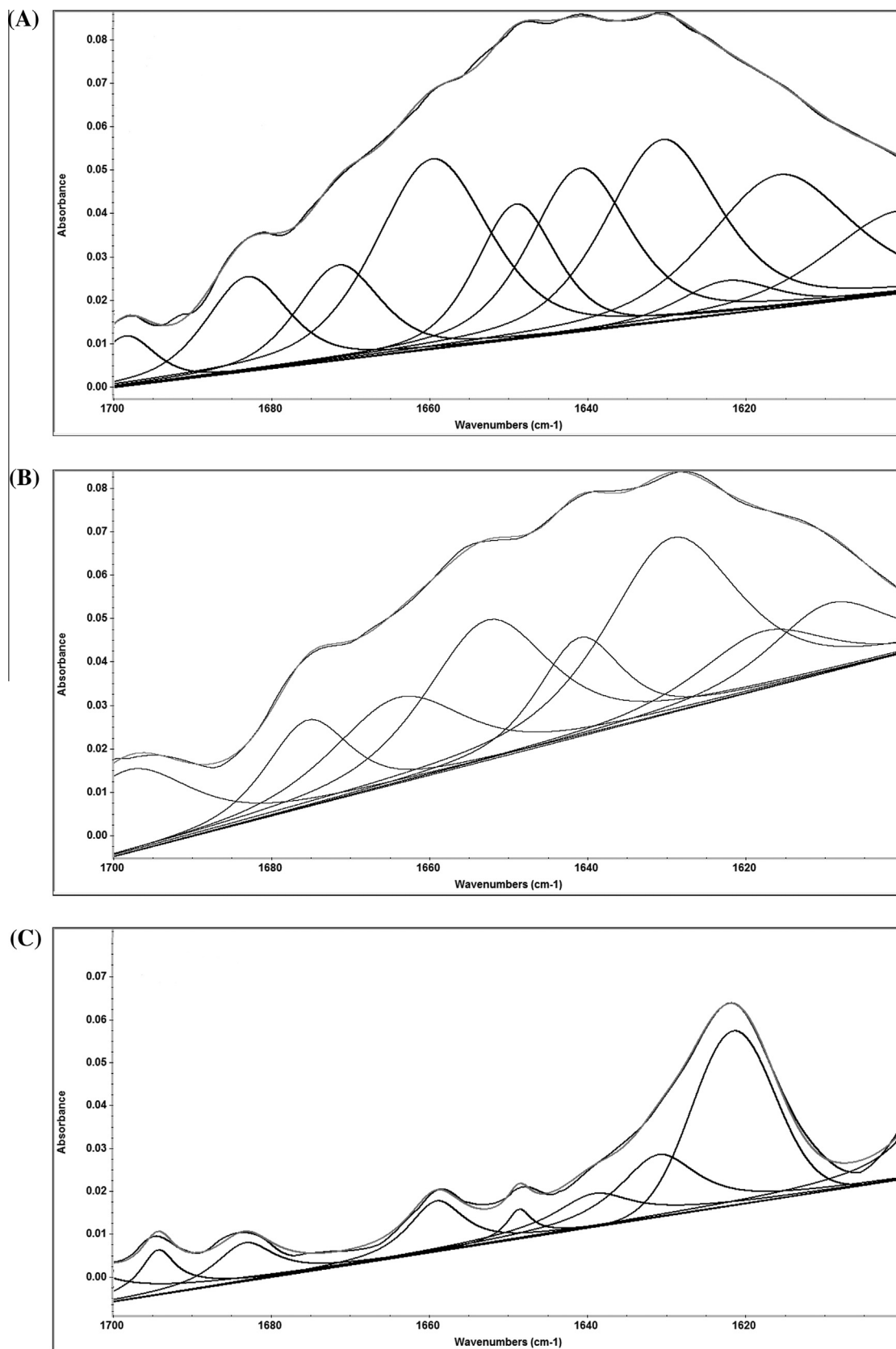


Fig. 6. (A) The original and curve-fitted FT-IR spectrum of: (A) native papain; (B) thermally treated papain and (C) cold stored papain.

Renaturation curves were also recorded but no significant change in the extinction coefficient was detected, so we concluded that thermal denaturation process is irreversible (data not shown).

It is well known that papain family of proteases are secreted as zymogens, thus the cleavage of the activation peptide could affect papain renaturation [2] especially as propeptide can play a role of

Table 3

Assignments and relative band areas of Amide I components of native, thermally treated and cold stored papain. Relative band areas were determined by curve-fitting analysis. Inter – intermolecular; Intra – intramolecular β -sheet.

Native papain		Thermally treated papain		Cold stored papain		Assignment
ν (cm^{-1})	Area (%)	ν (cm^{-1})	Area (%)	ν (cm^{-1})	Area (%)	
1623	3.6	1618	9.1	1621	38.7	Inter/Intra β [24–27]
1630	22.8	1628	27.2	1629	19.2	Inter β -sheet [24]
1641	19.8	1641	9.7	1639	11.7	Random coil [24]
1648	10.8	–	–	1648	3.4	α -Helix [24]
1659	21.3	1653	20.6	1659	10.7	α -Helix [24]
1672	9.9	1974	8.2	–	–	Unordered helix [25]
1683	8.5	1663	13.0	1683	8.8	Turn [24]
1697	3.3	1698	12.2	1695	7.5	Inter/Intra β [24–26]

Table 4

Fitted values for the secondary structures present in the native, thermally treated and cold denatured papain determined by ATR FT-IR spectroscopy and secondary structure contents calculated from X-ray analysis.

Papain sample	β -sheet	Secondary structure content (%)				
		α -helix	Unordered structures	Turn	Inter/Intramolecular β -sheet	RMS error
Native	22.8	32.1	29.7	8.5	6.2	3.5
Thermally treated	27.2	20.6	17.9	13.0	21.3	0.1
Cold stored	19.2	11.7	14.1	8.8	46.2	1.4
X-ray	25	26	42	7	–	–

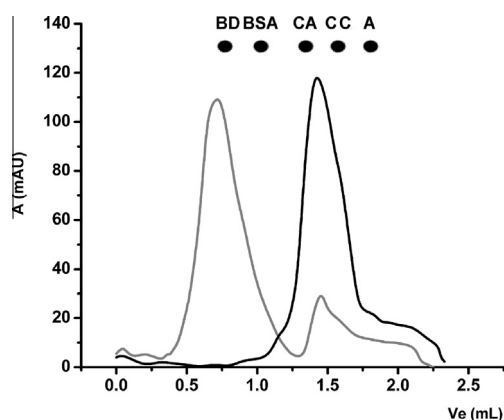


Fig. 7. Size exclusion chromatography analysis of native (black line) and cold stored papain (gray line). BD, BSA, CA, CC and A – gel filtration molecular weight standards (blue dextran 2000 kDa, bovine serum albumin 67 kDa, carbonic anhydrase 29 kDa, cytochrome c 14 kDa and aprotinin 6.5 kDa, respectively).

intramolecular chaperonin ensuring the correct folding of the molecule [33].

FT-IR spectroscopy is well established method for the analysis of protein secondary structure. The mostly used spectral region for secondary structure analysis is amide I band (frequency limits: 1600–1700 cm^{-1}), which is due almost entirely to the C=O stretch vibrations of the peptide bonds [34]. Recent studies showed that specific low frequency bands present in the amide I region of FT-IR second derivate spectrum appear when analysed proteins tend to aggregate. Several authors reported that frequencies 1614–1624 cm^{-1} and above 1684 cm^{-1} are aggregation specific frequencies, and they appear due to the formation of intermolecular antiparallel β -sheets prior to aggregation [26,27,35]. Two peaks at the frequency as low as 1621 cm^{-1} and as high as 1695–1698 cm^{-1} that could be observed in the spectrum of thermally treated and cold stored papain (Fig. 6B and C, respectively) we assigned to aggregation specific intermolecular antiparallel β -sheet. However, we detected similar bands in the spectrum of native papain as well, but their contribution was in total 6.2% in comparison to 21.3% and 46.2% of the total amide I area found in

the case of thermally treated and cold stored papain, respectively (Table 4). The presence of papain aggregates formed upon cold storage was confirmed by analytical size exclusion chromatography (Fig. 7). Just a minor portion of native papain could be detected in the chromatogram of cold stored protein explaining such a dramatic activity loss after six freeze–thaw cycles (Fig. 3). Monitoring of papain autoproteolysis by CBB binding and RP-HPLC (Fig. 4) suggests that detected inactivation of 75% (Fig. 3) cannot be attributed to papain autoproteolysis.

A theory behind denaturation of proteins in the frozen state claims destabilization of protein native structure since hydrophobic interactions become weaker with decreasing temperature which leads to the exposure of non-polar residues to water accounting for unfolding [36]. Several authors had reported the increase of β -sheet content in cold denatured proteins compared to the native ones [37,38]. Furthermore, computer simulations have shown that this could be related to formation of a thin layer of water around non-polar residues that is favorable at low temperatures. This thin layer can be accommodated between residues that form β -sheet structures but not α -helix [39,40]. Since cold denatured states tend to favor the formation of β -sheets which are building blocks of fibril-like aggregates [41] it could be of importance for protein activity loss during thawing or lyophilisation.

Denaturation and aggregation of proteins during cold storage, especially the ones with commercial application is of particular concern to ever growing polypeptide based industry [42]. Despite of general belief that the activity loss detected upon the cold storage of proteases happens as a consequence of autoproteolysis, in this work it is undoubtedly shown that upon the cold storage papain underwent structural rearrangements and aggregation that correspond to other cold denatured proteins, rather than autoproteolysis.

Studying cold stability of industrially important proteases, including papain, could lead to development of better preservation methods (the usage of cryoprotectants) and prolonged shelf life.

Acknowledgement

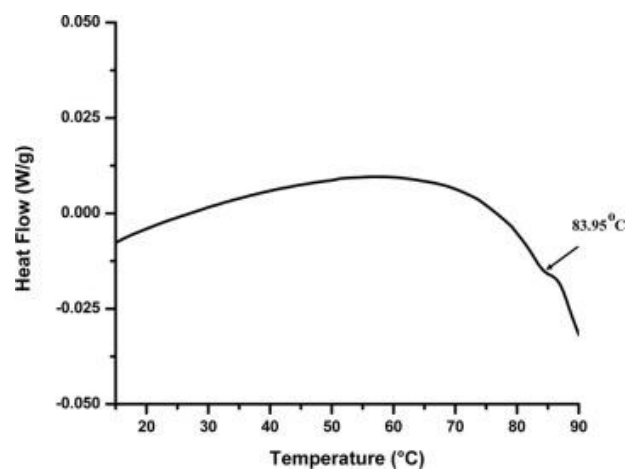
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.saa.2015.05.061>.

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Supplementary figure S1. DSC thermogram of papain.



Regular article

Optimizing storage conditions to prevent cold denaturation of trypsin for sequencing and to prolong its shelf life



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ABSTRACT

Trypsin is a serine protease with widespread applications, including protein sequencing and trypsin mass fingerprinting. In the present study, the storage of trypsin in acidic conditions significantly affected the recovery of activity (40%) after 7 freeze–thaw cycles. Further, trypsin lost parts of its native secondary structure elements, which resulted in a 10% increase in β -sheet content (band maximum detected at a frequency of 1634 cm^{-1} in the Fourier transform infrared (FT-IR) spectrum) indicative of freezing-induced denaturation of the protein. The cold storage of trypsin in ammonium bicarbonate (pH 8.2) with the addition of cryoprotectants, such as glycerol or lysine, led to protein stabilization (complete secondary structure content preservation was detected by FT-IR), higher activity recovery (>90%) and modest autolysis (<10%). High activity recovery (>90%) was also detected with the addition of propylene glycol and polyethylene glycol, saccharides and arginine. Nevertheless, trypsin stored at pH 8.2 with the addition of glycerol or lysine was as efficient as untreated trypsin in the trypsin mass fingerprinting analysis of BSA, suggesting that the cold storage of trypsin in slightly alkaline conditions with the addition of cryoprotectants could prolong its shelf life.

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1. Introduction

Trypsin is a serine protease excreted by pancreatic cells. The enzyme is synthesized as a zymogene [1]. It cleaves peptide bonds after the positively charged amino acids lysine (K) or arginine (R) [2]. The optimal pH value for trypsin activity is slightly alkaline (approximately 8) [3]. Some of its widespread applications include: laboratory and industrial protein digestion [4], adherent cell detachment [5] and, in recent decades, trypsin has been used as an enzymatic tool for protein sequencing and trypsin mass fingerprinting [6]. Proteases rapidly lose their activity during cold storage in solution and major manufacturers recommend storing them in the lyophilized form. However, general laboratory practice proposes the storage of cell culture trypsin and proteomic trypsin in a soluble form. Manufacturers recommend reconstitution of lyophilized proteomic or sequencing grade enzymes in acidic

conditions (hydrochloric or acetic acid) to prevent massive autolysis [1]. Such storage is limited to just a few freeze–thaw cycles.

During freezing and storage of proteins in the frozen state, several factors can impact the protein stability. Cold denaturation of globular proteins has been detected in several cases, especially at high pressure [7–9]. Theoretically, cold denaturation should happen as a consequence of changes in the interactions between water and protein molecules at low temperatures and should represent a universal phenomenon. However, cold denaturation is difficult to study because most proteins have denaturation points below 0°C [10]. Furthermore, the denaturation of protein during freezing could also occur as a consequence of changes in the solute concentration due to ice formation, exposure of protein to the ice–liquid interface and changes of the pH value surrounding the protein [11]. Freeze–thaw cycles are routinely used as a tool to determine the combined effects of these different freezing related stresses on protein stability [11,12].

Trypsin can be reversibly inactivated at acidic pH values [1]. In addition to manufacturers' recommendations for trypsin cold storage, we could not find novel literature dealing with trypsin cold

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Table 1
List of cryoprotectants used to stabilize trypsin.

Additives—polyols and polymers	Final concentration
Propylene glycol (PG)	0.3 M
Glycerol (G)	0.3 M
Polyethylene glycol 6000 (PEG)	0.002 M
Additives—saccharides	
Glucose (Glc)	0.05 M
Sucrose (Sacc)	0.03 M
Lactose (Lac)	0.03 M
Additives—amino acids	
Lysine (K)	0.1 M
Arginine (R)	0.1 M

stability, especially published in recent decades, in which elucidation of the mechanism and understanding of the cold denaturation phenomenon are the focus. However, there are numerous reports dealing with the cold stability of proteins of interest for the pharmaceutical industry [13,14]. The addition of polyols, saccharides and free amino acids could stabilize proteins during cold storage or lyophilization [11,13–15].

Some interesting questions arise out of this problem. Are acidic conditions the best for trypsin cold storage? Could the storage of an enzyme in conditions far from its optimal conditions cause an increase of inactivation due to cold denaturation to a greater extent than the loss of activity due to autolysis?

The aim of this paper was to determine the optimal conditions for sequencing grade trypsin cold storage in the soluble form. Our specific aim was to check the postulated hypothesis that trypsin could be structurally more stable at its operating pH values than in acidic conditions during freeze–thawing. In the present study, the effects of acidic and slightly alkaline conditions were compared in terms of the trypsin activity recovery, secondary structure content and autolysis. Further, the ability of cryoprotectants (polyols, polymers, saccharides and amino acids) to stabilize trypsin during freeze–thawing was also examined.

2. Material and methods

2.1. Materials

Bovine trypsin for sequencing, $N\alpha$ -benzoyl-L-arginine *p*-nitroanilide (BAPNA), blue dextran, bovine serum albumin (BSA), carbonic anhydrase, cytochrome *c* and MS grade ammonium-bicarbonate were purchased from Sigma–Aldrich, Steinheim, Germany. All other chemicals were of analytical grade and were used without further purification.

2.2. Enzyme solutions

The influence of additives on trypsin cold stability in slightly alkaline conditions was tested in 100 mM ammonium-bicarbonate in 1 mg/mL of trypsin. The influence of additives on trypsin cold stability in acidic conditions was tested in 1 mM HCl in 1 mg/mL of trypsin. Three different classes of additives were used (Table 1).

2.3. Freeze–thaw cycles

Trypsin for sequencing (1 mg/mL) dissolved in acidic or alkaline conditions was frozen at -20°C . The freezing rate was 1°C per minute. After one hour, the samples were thawed. The thawing rate was $>10^{\circ}\text{C}$ per minute. Slow freezing and fast thawing process (up to 25°C) was performed as suggested [16] in order to minimize damage to trypsin. Freeze–thaw cycles were repeated up to seven times.

For the activity measurements, an aliquot was taken after each cycle and diluted 100 times in 100 mM Tris buffer pH 8.0. The aliquot was taken immediately after disappearance of ice to limit sample incubation at above zero to less than 2 min per cycle.

2.4. Enzyme activity assay

The enzymatic activity of trypsin stored under different conditions was tested using BAPNA as a substrate in a 96-well microplate (Sarstedt, Numbrecht, Germany) as described [17], with some modifications. Briefly, 50 μL of 100 times diluted enzyme solution was added to 100 μL of substrate solution (1 mM BAPNA in 100 mM Tris buffer pH 8.0). After 60 min of incubation at 25°C , the reaction was stopped by the addition of glacial acetic acid, and the absorbance at 405 nm was measured. Each enzyme aliquot was assayed in triplicate. The relative activity of trypsin after each cycle was considered as the activity remaining in the sample. The starting activity detected in the sample was considered as 100%.

2.5. Trypsin autolysis

2.5.1. Autolysis measurement

Autolysis was measured as described previously [18,19], with some modifications. To test the autolysis in slightly alkaline conditions, trypsin was dissolved in 100 mM ammonium-bicarbonate as a control or in 100 mM ammonium-bicarbonate containing 0.3 M glycerol (AB-G) or 0.1 M lysine (AB-K) to a concentration of 1 mg/mL. To test the autolysis in acidic conditions, trypsin was dissolved in 1 mM HCl. An aliquot was immediately tested. Solutions were subjected to 7 freeze–thaw cycles as already described. After each cycle, an aliquot (5 μL) was mixed with 200 μL of CBB (0.125% Coomassie Brilliant Blue G-250 in 24% ethanol and 48% phosphoric acid). After 5 min, the absorbance at 595 nm was measured. The assay was performed in triplicate.

2.5.2. Gel filtration and SDS PAGE analysis of autolytic products

Analytical gel filtration of the untreated trypsin and freeze–thawed trypsin samples stored in acidic conditions (in 1 mM HCl) or in alkaline conditions (100 mM ammonium-bicarbonate as a control and in 100 mM ammonium-bicarbonate containing 0.3 M glycerol or 0.1 M lysine) was performed in 100 mM Na-phosphate buffer pH 7.4 using an Äkta Purifier 10 System (GE Healthcare, Uppsala, Sweden) on a Superdex 75 PC 3.2/30 column according to the manufacturer's instructions. To prevent further autolysis during chromatography, all samples were treated with 1 mM PMSF (phenylmethylsulfonyl fluoride) before loading on the column. The calibration of the column was performed using a mixture of gel filtration molecular weight standards (1 mg/mL each): blue dextran, bovine serum albumin, carbonic anhydrase and cytochrome *c*.

SDS PAGE was performed in protein electrophoresis unit Hoefer Dual Gel Caster Mighty small SE 245 system (Hoefer, Holliston, MA, USA) according to manufacturer's instructions using 12% resolving gel and 4% stacking gel.

2.6. Sample preparation for Fourier transform infrared spectroscopy and BSA digestion

Four trypsin samples were prepared for secondary structure determination and BSA digestion followed by LC–MS analysis.

Untreated trypsin was prepared by the manufacturer's instructions by dissolving the enzyme to a concentration of 1 mg/mL in 1 mM HCl. Fourier transform infrared (FT-IR) spectra were collected (as described in Section 2.7.1), and BSA digestion was performed immediately (as described in Section 2.8.1).

Cold stored trypsin in HCl was prepared by the manufacturer's instructions by dissolving the enzyme to a concentration of 1 mg/mL in 1 mM HCl, and it was then subjected to seven freeze–thaw cycles as already described.

Cold stored trypsin in AB-G or AB-K was prepared by dissolving the enzyme to a concentration of 1 mg/mL in 100 mM ammonium-bicarbonate containing 0.3 M glycerol (AB-G) or 0.1 M lysine (AB-K) and then freeze–thawed for seven times as previously described.

After seven freeze–thaw cycles, the trypsin samples were used for BSA digestion (as described in Section 2.8.1), and FT-IR spectra were recorded (as described in Section 2.7.1).

2.7. Fourier transform infrared spectroscopy

2.7.1. Collection of spectra

Infrared spectra were gathered for both untreated and cold stored trypsin using FT-IR with attenuated total reflectance (ATR) at 1 cm⁻¹ resolution (Nicolet 6700 FT-IR, software OMNIC, Version 7.0, Thermo Scientific, USA). The enzyme solution (10 µL) was applied onto a Smart accessory with a diamond crystal (Smart Orbit, Thermo Scientific, USA). The solvent was evaporated by a nitrogen stream to obtain a thin ATR film. For each spectrum, 25 scans were collected. The spectrum of the respective buffer was subtracted from the spectrum of the protein because it interferes with the protein absorbance in the amide I region. After subtraction, the spectrum was smoothed to remove the noise (factor of 6.75 cm⁻¹). Criteria for the correctness of subtraction were the removal of the band near 2200 cm⁻¹ and a flat baseline between 1800 and 2000 cm⁻¹ for samples in water. For the sample stored in AB-K, additional correction of the lysine band in the amide I region was necessary.

2.7.2. Secondary structure analysis

The amide I region (1700–1600 cm⁻¹) was decomposed to its constituents that would be assigned to certain structural features. The individual peak frequency position and width were obtained from the second derivative spectra. The contribution of each peak to the amide I band was assessed by integrating the area under the curve and then normalizing it to the total area under the amide I band.

Assignment of the secondary structures for all observed peaks was performed according to guidelines published previously [20,21]. The frequency limits under Lorentzian band shapes were as follows: 1651–1656 cm⁻¹, α -helix; 1689–1673 cm⁻¹ and 1637–1620 cm⁻¹, β -sheet; 1673–1662 cm⁻¹, turns; 1651–1637 cm⁻¹ and 1662–1656 cm⁻¹, unordered structures.

2.7.3. IR spectral correlation

To examine the structural similarity of untreated trypsin dissolved in HCl and trypsin samples subjected to 7 freeze–thaw cycles, the comparison of second derivative spectra was applied as previously described [22,23]. The spectral correlation coefficient was calculated using the equation:

$$r = \frac{\sum_{i=1}^n x_i y_i}{\sqrt{\sum_{i=1}^n x_i^2 y_i^2}} \quad (1)$$

where x_i and y_i represent the spectral absorbance data of the reference and sample spectra at the i -th frequency position. The calculation were performed in the amide I region.

2.8. Trypsin mass fingerprinting

2.8.1. BSA digestion

BSA digestion was performed according to the manufacturer's instructions. Briefly, BSA (1 mg) was incubated in 100 µL of 8 M

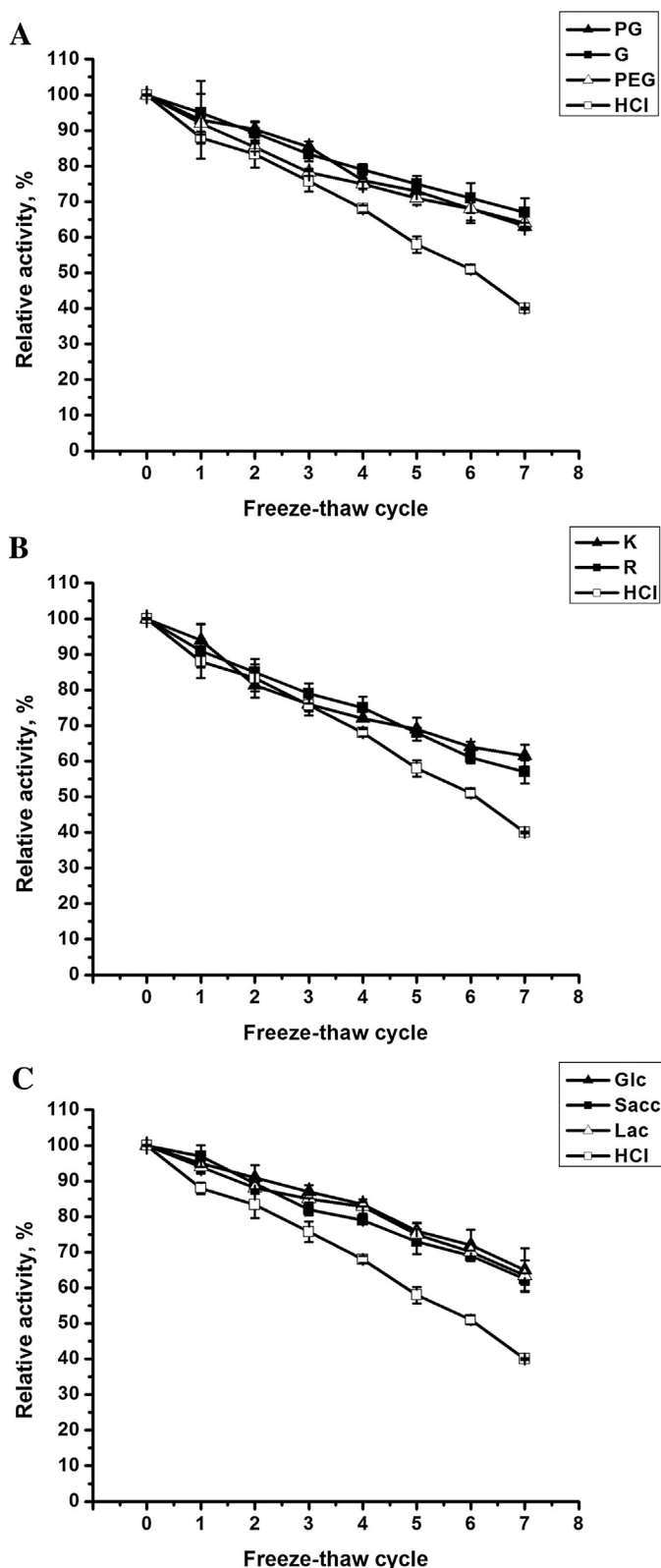


Fig. 1. The influence of cryoprotectants on the remaining trypsin activity after freeze–thawing in 1 mM hydrochloric acid (HCl), as recommended by manufacturer: (A) PG—propylene glycol, G—glycerol, PEG—polyethylene glycol; (B) K—lysine, R—arginine and (C) Glc—glucose, Sacc—sucrose, Lac—lactose, HCl—1 mM HCl only. Data represents mean values \pm standard deviation of three independent experiments.

urea and reduced by dithiothreitol (DTT). The BSA solution was diluted 10 times using 50 mM ammonium-bicarbonate. Afterwards, untreated trypsin or cold stored trypsin in HCl, AB-G or AB-K was added to obtain a BSA:trypsin ratio 150:1 (w/w). The digestion proceeded for 12 hrs at 37 °C.

2.8.2. Liquid chromatography–mass spectrometry (LC–MS) analysis

The obtained peptides were injected onto a reversed-phase C18 column (RR HT, 1.8 μ m, 4.6 \times 50 mm) coupled with a Zorbax Eclipse XDB-C18 installed on a 1200 series HPLC system (Agilent Technologies). The sample was separated using an acetonitrile gradient (5–95% for 10 min with 0.2% formic acid, 95% for 5 min). The mass spectrometer (6210 Time-of-Flight (TOF) LC–MS system (G1969A; Agilent Technologies, Santa Clara, CA, USA)) was run in positive electron spray ionization (ESI) mode with a capillary voltage of 4000 V, fragmentor voltage of 200 V and mass range of 100–3200 m/z . The peptide masses were searched against the SwissProt protein sequence database using the MASCOT program.

2.9. Determination of activation energy for deactivation

Activation energy of cold denaturation was determined using Borchardt–Daniels method [24].

Borchardt and Daniels approach assumes that the reaction follows n -th order kinetics and obeys the general rate equation:

$$\frac{d\alpha}{dt} = k(T)(1 - \alpha)^n \quad (2)$$

where $d\alpha/dt$ is the reaction rate, α is the reaction conversion degree, $k(T)$ is the specific rate constant at temperature T and n is the order of the reaction [24].

Assuming that the reaction follows Arrhenius behavior [25]:

$$k(T) = Ze^{-\frac{E_a}{RT}} \quad (3)$$

logarithm of Eq. (2) yields:

$$\ln\left(\frac{d\alpha}{dt}\right) = \ln(Z) - \frac{E_a}{RT} + n\ln(1 - \alpha)$$

$$\ln(k(T)) = \ln\left(\frac{d\alpha}{dt}\right) - n\ln(1 - \alpha)$$

Combining these two:

$$\ln(k(T)) = \ln(Z) - \frac{E_a}{RT}$$

plot of $\ln(k(T))$ versus $1/T$ for at least 20 points for $0.1 < \alpha < 0.5$, for the set value of n , should exhibit linear dependence, yielding the value of the activation energy as the slope (E_a/R) and the pre-exponential factor $\ln(Z)$ as the intercept [24].

3. Results and discussion

3.1. The effect of additives on trypsin activity during freeze–thawing

The storage of trypsin in acidic conditions (1 mM HCl, as recommended by the manufacturer) led to massive loss of activity after freezing and thawing. After 7 cycles, approximately one third of the initial activity could be detected (Fig. 1).

In order to test the storage of trypsin in alkaline conditions, we decided to choose AB buffer because both ammonium and bicarbonate ions are volatile and should not influence the MS analysis. The storage of trypsin in slightly alkaline conditions (100 mM ammonium-bicarbonate) provided better preservation of activity in comparison to the samples stored in HCl (Fig. 2).

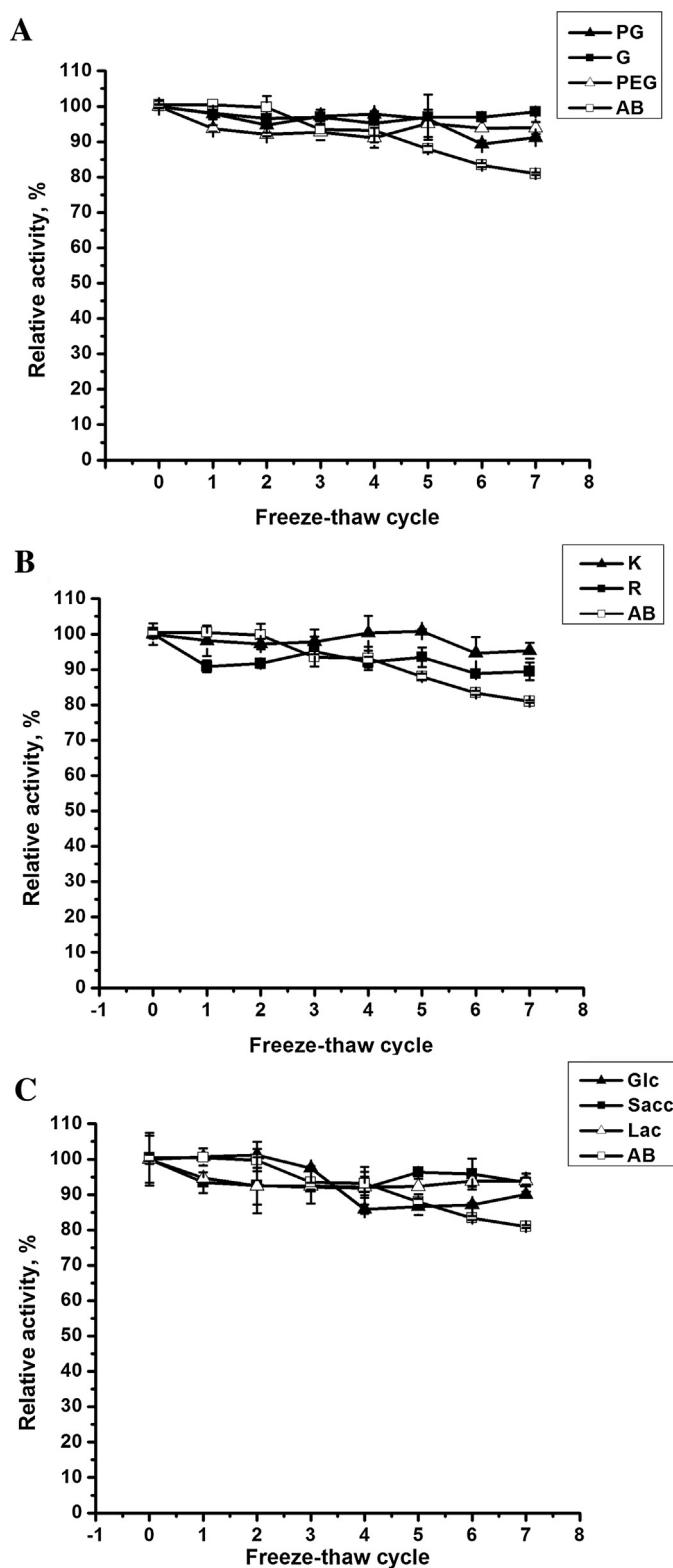


Fig. 2. The influence of cryoprotectants on the remaining trypsin activity after freeze–thawing in 100 mM ammonium-bicarbonate (AB): (A) PG–propylene glycol, G–glycerol, PEG–polyethylene glycol; (B) K–lysine, R–arginine and (C) Glc–glucose, Sacc–sucrose, Lac–lactose, AB–100 mM AB only. Data represents mean values \pm standard deviation of three independent experiments.

Table 2

Fitted values for the secondary structures present in the trypsin samples determined by ATR FT-IR spectroscopy. The secondary structure content calculated from the X-ray analysis is given. Samples: trypsin dissolved in 1 mM HCl (untreated) and trypsin samples after 7 freeze–thaw cycles: dissolved in 1 mM HCl (HCL); dissolved in 100 mM ammonium-bicarbonate with addition of 0.3 M glycerol (AB-G) or 0.1 M lysine (AB-K).

Sample	β -Sheet, %	α -Helix, %	Turn, %	Unordered, %	RMS error, %
Untreated	48.2	6.5	16.4	28.9	3.7
HCL	58.0	5.6	13.9	22.4	2.1
AB-G	47.5	8.2	14.3	30.0	3.2
AB-K	46.3	7.8	14.1	31.8	5.3
X-ray	46.2	8.1	12.1	33.6	–

The stabilizing effect was found in the case of all tested additives in both acidic and slightly alkaline conditions (Fig. 1 and 2). The additive-induced stabilization of trypsin stored in acidic conditions was around 20% in the case of all tested cryoprotectants and could not overcome the destabilizing effect of a low pH value which was around 65% (Fig. 1). At slightly alkaline conditions, polyols and polymers increased trypsin stability, which was found in the case of glycerol to be almost the same as the starting activity after 7 freeze–thaw cycles ($97 \pm 1\%$). Propylene glycol and polyethylene glycol stabilized trypsin to the same extent (93% of activity remaining after 7 freeze–thaw cycles) (Fig. 2A).

Results of glycerol effect on trypsin activity recovery after freezing–thawing are in a good agreement with the previous literature. In the study of Koseki et al. 0.3% (v/v) glycerol has been shown to prevent cold denaturation of ovalbumin [26]. Glycerol at concentration 1 M also enabled activity recovery of catalase from 80 to 95% [27].

Both lysine (relative activity measured after 7 freeze–thaw cycles was $95 \pm 2\%$) and arginine ($89 \pm 2\%$) stabilized trypsin in the tested concentrations (Fig. 2B). The rationale of using lysine and arginine out of the 20 natural amino acids was the assumption that K and R could hamper trypsin autolysis because they are amino acids that are recognized by trypsin in the substrate binding pocket [2].

Lysine and arginine are positively charged amino acids, and similarly as suggested in the case of negatively charged glutamate, it could be assumed that K and R can associate non-specifically to the protein surface and increase its positive charge which leads to increased protein hydration. This effect can prevent alteration of protein's conformation and aggregation upon water removal from the protein surface during freezing [15]. Furthermore, arginine is one of the most commonly used additive for prevention of non-covalent protein aggregation [28].

Saccharides stabilized trypsin in slightly alkaline conditions (Fig. 2C) to the same extent (around 90% of starting activity was detected after 7 freeze–thaw cycles). Studies of influence of saccharides on protein stability and activity during freezing–thawing were conducted on many other model proteins showing saccharides' stabilizing effect. For example, it has been shown LDH lost substantial percentage of activity when freezing in buffer, while it was stabilised with addition of sugars and PEG [29].

Polyols, saccharides and amino acids are known stabilizers of proteins in solution. These additives are considered to be preferentially excluded from the protein surface, therefore favouring its folded state [30]. They serve to prevent protein damage during freeze–thawing by protecting the protein surface from freezing-induced stresses, such as changes in the concentration of solutes, alteration of the pH and sub-zero temperatures [13]. In the case of PEG, despite its binding to the protein at higher temperatures ($>25^\circ\text{C}$), lowering the temperature weakens the hydrophobic interaction, so even PEG became preferentially excluded from the protein surface and protected the protein by the same mechanism

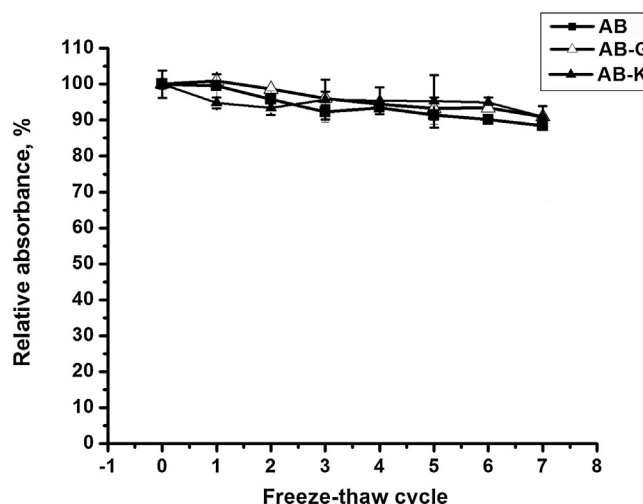


Fig. 3. Autolysis of trypsin dissolved in 100 mM ammonium-bicarbonate (AB) during 7 freeze–thaw cycles. The absorbance of each sample is shown as a percentage of the initial absorbance. AB-G—AB with 0.3 M glycerol; AB-K—AB with 0.1 M lysine. Data represents mean values \pm standard deviation of three independent experiments.

as polyols, saccharides and amino acids [31]. The same classes of additives were proved to increase the recovery of protein activity after lyophilization [27].

Because our main goal was to optimize the buffer composition to preserve trypsin activity after several freeze–thaw cycles for application in proteomic techniques, we chose to proceed with 100 mM ammonium-bicarbonate containing 0.3 M glycerol or 0.1 M lysine as the stabilizers providing the highest recovery of activity. Further, we wanted to investigate the causes of such dramatic activity loss detected in acidic conditions that could not be overcome in the presence of tested additives.

3.2. Trypsin autolysis

In order to exclude possible contribution of autolysis to activity loss in both alkaline and acidic conditions, we monitored trypsin autolysis during 7 freeze–thaw cycles in the assay based on CBB binding. Furthermore, to estimate the damage to trypsin, analytical gel filtration and SDS PAGE were performed. The results are shown in Figs. 3 and 4 and Supplementary Fig. S1.

Trypsin stored in acidic conditions was less prone to autolysis when compared to trypsin stored in bicarbonate. Autolysis of trypsin in 1 mM HCl in the CBB-binding based assay was not detected (data not shown), whereas in the gel filtration, a small aggregate-specific peak appeared (Fig. 4). In the case of 100 mM ammonium-bicarbonate alone (AB) or containing 0.3 M glycerol (AB-G) and 0.1 M lysine (AB-K), after seven freeze–thaw cycles, less than 10% of the CBB-binding was lost due to autolysis (Fig. 3). In gel filtration and SDS PAGE, the trypsin peak/band was identified at a molecular weight of 23 ± 1 kDa in all samples, and its preservation implies very modest autolysis of cold-stored trypsin at alkaline pH values (Fig. 4 and Supplementary Fig. S1). Calibration curve for molecular weight determination in gel filtration is presented in Supplementary Fig. S2.

The autolysis rate of trypsin at pH 8 has been described elsewhere (e.g., measured at 25°C , trypsin autolysis was limited to a few percent in the first 10 min) [32,33].

The low molecular weight peak (<12 kDa) could be detected in the untreated sample, as well as in trypsin stored in acidic conditions (probably fragments or impurity present in commercial preparation). However, this peak disappeared after storage in slightly alkaline conditions, probably due to digestion by active

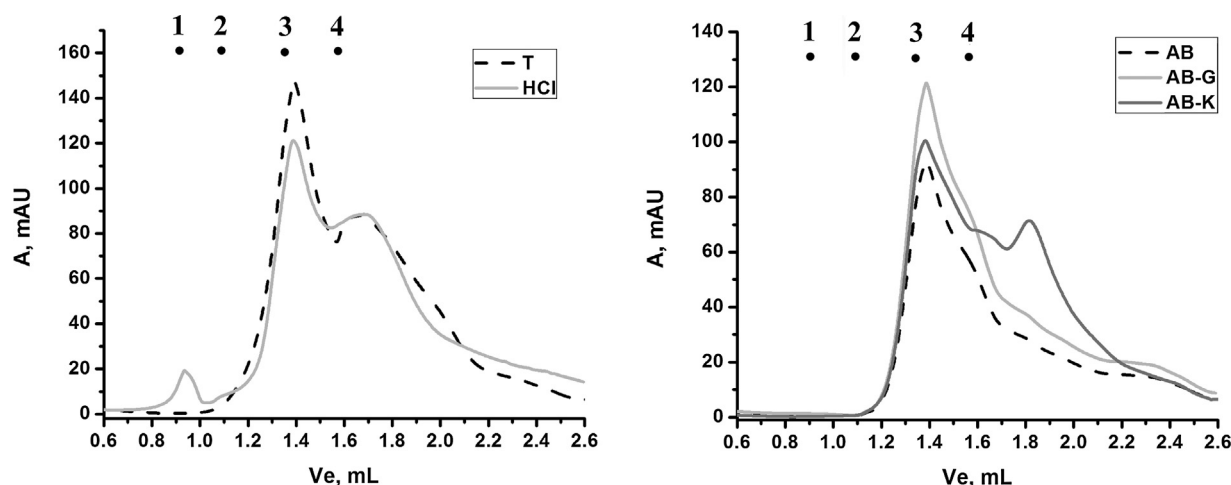


Fig. 4. Gel filtration analysis of the untreated trypsin (T) and trypsin samples after 7 freeze–thaw cycles. (A) Trypsin dissolved in 1 mM HCl and (B) trypsin dissolved in 100 mM ammonium-bicarbonate (AB) with 0.3 M glycerol (AB-G) and 0.1 M lysine (AB-K). Points 1–4, gel filtration molecular weight standards (blue dextran, bovine serum albumin, carbonic anhydrase and cytochrome c, respectively).

trypsin, except in the presence of 0.1 M lysine. Partial disappearance of low molecular weight bands in alkaline samples (<12 kDa) was shown in SDS PAGE as well, except in the presence of 0.1 M lysine (Supplementary Fig. S1). The recommendation of manufacturers to store trypsin at low temperature in acidic conditions is based on its low autolysis rate at pH 3 [1]; however, despite the prevention of autolysis, such a protocol is not well-suited for storage of proteomic trypsin because during each freeze–thaw cycle, approximately 9% of activity is lost (Fig. 1). On the other hand, the activity loss detected in trypsin stored in ammonium-bicarbonate with the addition of glycerol or lysine (Fig. 2) and trypsin autolysis (Figs. 3, 4 and Supplementary Fig. S1) was limited to just a few percent, suggesting that the storage of trypsin for proteomics at optimal pH could prolong its shelf life.

3.3. Changes in the secondary structure contents of freeze–thawed trypsin

Because the observed loss of activity in acid-stored trypsin could not be attributed to autolysis, we employed FT-IR to determine whether there were any perturbations in the secondary structure of the trypsin samples.

Changes in the amide I region of the IR spectra of trypsin dissolved in 1 mM HCl induced by 7 freeze–thaw cycle are presented in Fig. 5. Fig. 5A shows the original and second-derivative IR spectra of untreated trypsin dissolved in acid. The major peaks assigned to β -sheet were 1680.6 and 1635 – 1631 cm^{-1} . Among the unordered structures, random coils were represented with peaks at 1648.9 and 1643.1 cm^{-1} , and the peak found at 1659.7 cm^{-1} was assigned to unordered helix. Ordered helix peak can be observed only in the second derivative spectrum (1653 cm^{-1}). The spectra of the trypsin freeze–thawed in acidic conditions (Fig. 5B) showed a shift to lower frequencies, with the maximum at 1634.4 cm^{-1} , indicating increased content of β -sheet structure, as well as less pronounced peaks assigned to the unordered structures.

The original and second-derivative spectra (amide I region) of the trypsin samples dissolved in 100 mM ammonium-bicarbonate with the addition of 0.3 M glycerol (Fig. 6A) or 0.1 M lysine (Fig. 6B) did not differ much with respect to the intensity and the position of the major peaks in both spectra. The most prominent peaks were assigned to unordered structure and β -sheets. However, by simple introspection of the original IR spectra of trypsin at pH 3 (Fig. 5A) and pH 8 (Fig. 6A and B), an increase of the peak at 1634 cm^{-1} occurred, even without freeze–thawing. It is possible

that even a simple incubation at acidic pH values induced conformational changes in trypsin [34] that could explain the difference in the spectra. However, the increase of the 1634 cm^{-1} peak is even more pronounced after 7 freeze–thaw cycles (Fig. 5B). Similar spectra, with the most pronounced three maxima in the region 1630 – 1660 cm^{-1} , were recorded for trypsin under acidic conditions [35], whereas the spectra recorded at near neutral pH values had only two maxima [36].

After decomposition of the amide I region of the IR spectra, peaks were assigned to specific secondary structures, and the relative percentage of each secondary structure was calculated using the contribution of the integrated intensities of the band areas to the total amide I band area. The content of the secondary structures in the X-ray determined tertiary structure of trypsin was also calculated from the UniProt data bank entry (P00760 (TRY1_BOVIN)). The results are presented in Table 2.

The content of the secondary structures determined here were similar to those calculated from the X-ray structure of trypsin in the case of untreated trypsin and trypsin stored in alkaline conditions. A significant increase of the β -sheet content and decrease of the α -helical content and unordered structures was found in the case of the trypsin sample freeze–thawed for 7 cycles at pH 3 (Table 2). However, in comparison to the X-ray data and the secondary structures content of the trypsin samples dissolved in 100 mM ammonium-bicarbonate, some insignificant changes of the secondary structures were also present in the untreated sample (trypsin dissolved in 1 mM HCl), which is in agreement with the slight increase of the 1634 cm^{-1} peak in the control sample (Fig. 5A).

The calculated correlation coefficients indicate that, although every frozen–thawed sample was different from the control (correlation coefficients significantly less than 1.0), the spectra of trypsin dissolved in 100 mM ammonium bicarbonate were much more similar to the untreated trypsin spectra than the spectra of samples freeze–thawed in 1 mM HCl (Table 3).

The large difference in r values between the samples stored in alkaline conditions with additives and the samples stored in 1 mM HCl suggests that the maintenance of the secondary structures was notably higher if trypsin was frozen–thawed in the presence of additives in ammonium-bicarbonate. A similar approach was used by Prestrelski et al., who had also shown that additives for cryoprotection can significantly enhance the correlation coefficient of lyophilized and rehydrated lactate dehydrogenase when compared to its control in aqueous solution [22].

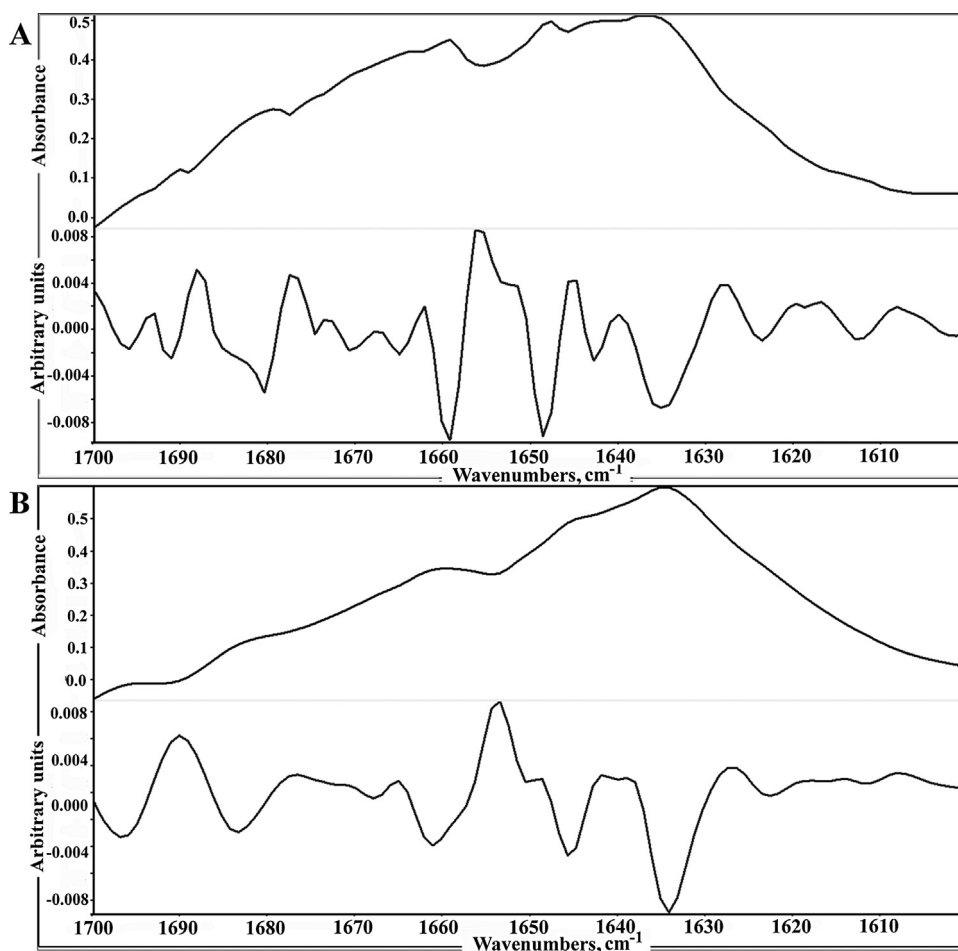


Fig. 5. The original (upper graph) and second-derivative (lower graph) Amide I region of the IR spectra of the acidic trypsin samples. (A) Trypsin dissolved in 1 mM HCl; (B) trypsin dissolved in 1 mM HCl after 7 freeze-thaw cycles.

Table 3

Spectral correlation coefficient for untreated trypsin dissolved in 1 mM HCl and samples after 7 freeze-thaw cycles: dissolved in 1 mM HCl (HCl); dissolved in 100 mM ammonium-bicarbonate with addition of 0.3 M glycerol (AB-G) or 0.1 M lysine (AB-K).

Sample	<i>r</i> Value
HCl	0.5536
AB-G	0.7452
AB-K	0.7991

Several authors have reported an increase of the β -sheet content in cold or acid denatured proteins compared to native proteins, sometimes followed by formation of aggregates [19,37,38]. Regarding the results presented in Fig. 5 and 6, Table 2 and 3, trypsin underwent structural changes consistent with those that occur in cold-denatured proteins. Cold denaturation of trypsin could provide an explanation for approximately 65% activity loss of trypsin dissolved in HCl after 7 freeze-thaw cycles (Fig. 1) and the appearance of aggregate peak of trypsin stored in acidic conditions (Fig. 4). We did not detect any cold-denaturation of trypsin samples freeze-thawed at pH 8.2. Furthermore, calculated activation energies for deactivation per freeze-thaw cycle were $21.4 \pm 0.2 \text{ kJ mol}^{-1}$ and $24.6 \pm 0.2 \text{ kJ mol}^{-1}$ in the case of trypsin dissolved in HCl and AB, respectively, while it was impossible to calculate energies in the presence of glycerol and lysine due to insignificant changes of activity per cycle within applied experimental setup. Such a result is not surprising because, in general, any factor that stabilizes protein in aqueous solution will have to tend the same effect during

freeze-thawing [13]; thus, storage of the trypsin at its operating pH value and in the presence of stabilizing additives should give the highest recovery of native protein after freeze-thawing, as has been shown in the cases of chymotrypsinogen [39] and lactate dehydrogenase [40].

3.4. Trypsin mass fingerprinting (TMF) of BSA and trypsin autolysis fragments detected by MS

BSA-specific peptides were identified in the samples digested with differently treated trypsin. According to Mascot, protein scores greater than 62 were considered as significant ($p < 0.05$). BSA identification by TMF was the most significant if untreated trypsin was used, giving the highest Mascot score and the lowest expectation value. Trypsin dissolved in 1 mM HCl and subjected to 7 freeze-thaw cycles was not as efficient in BSA digestion. Despite the detection of some BSA peptides, BSA was not detected as a significant hit. In the case of trypsin freeze-thawed in 100 mM ammonium-bicarbonate with additives, the scores and expectation values were similar to those obtained using untreated trypsin (Table 4). The list of detected peptides is shown in the Supplementary Table S1.

The trypsin autolysis fragments detected in the digested samples are presented in Supplementary Table S2. In all digested samples, with the exception of trypsin freeze-thawed in the presence of 0.1 M lysine, twelve matching peptides were found. Three of them (having monoisotopic masses: 632.320, 905.504 and 2551.248) were not detected in the trypsin sample with lysine as

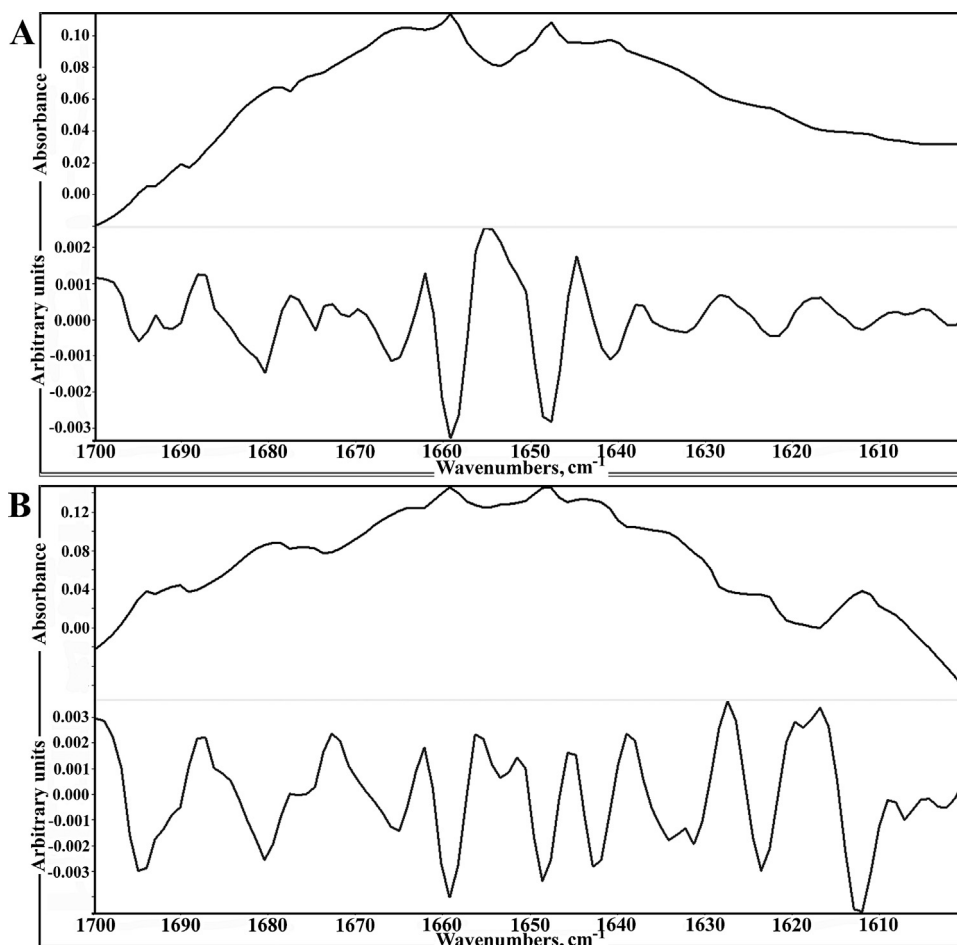


Fig. 6. The original (upper graph) and second-derivative (lower graph) Amide I region of the IR spectra of the trypsin dissolved in 100 mM ammonium-bicarbonate (AB) after 7 freeze–thaw cycles. (A) Trypsin dissolved in AB with 0.3 M glycerol (AB-G); (B) trypsin dissolved in AB with 0.1 M lysine (AB-K).

Table 4

Trypsin mass fingerprinting of BSA using trypsin samples: untreated trypsin and trypsin samples after 7 freeze–thaw cycles; dissolved in 1 mM HCl (HCL); dissolved in 100 mM ammonium-bicarbonate with addition of 0.3 M glycerol (AB-G) or 0.1 M lysine (AB-K).

Sample	Mascot score	Expect. value	Number of peptides	Coverage, %
Untreated	161	$5.3e^{-12}$	34	45
HCL	53	0.33	16	31
AB-G	159	$8.3e^{-12}$	34	44
AB-K	157	$1.3e^{-11}$	33	51

the additive. The absence of some autolytic peaks confirmed the expected autolysis suppression in the presence of lysine. Chamrád et al. recently showed that the presence of arginine led to a considerable decrease in bovine trypsin autolysis [41]. It could be that both lysine and arginine lower trypsin autolysis by occupying the substrate binding pocket in the trypsin molecule without causing a dramatic negative difference in the TMF (Table 2), taking the length of the digestion process into consideration [41].

4. Conclusions

During freeze–thawing of trypsin for sequencing, two main factors can impact the recovery of the activity: protein denaturation and autolysis. The present study reveals that significant changes in the secondary structure content of trypsin dissolved in acidic conditions (pH 3) after seven freeze–thaw cycles attributed to enlargement of the peak at approximately 1634 cm^{-1} assigned to β -sheet. Significant structural changes were not detected in trypsin

stored at pH 8.2 with the addition of the additives glycerol and lysine, implying that additional stabilization and an alkaline pH value inhibited the denaturation induced by freeze–thawing.

Regarding the concern of autolytic loss of trypsin activity, only modest autolysis was detected in ammonium-bicarbonate buffer systems by avoiding prolonged manipulation time with the samples at room temperature. Nevertheless, trypsin stored at pH 8.2 with the addition of glycerol or lysine was as efficient as untreated trypsin in the trypsin mass fingerprinting analysis of BSA, suggesting that the cold storage of trypsin in slightly alkaline conditions with the addition of cryoprotectants could prolong its shelf life.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bej.2015.09.018>.

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